PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 27 May 1999 (27.05.99)	
International application No. PCT/EP98/06040	**Applicant's or agent's file reference** FB/SD/B45110
International filing date (day/month/year) 17 September 1998 (17.09.98)	**Priority date** (day/month/year) 26 September 1997 (26.09.97)
Applicant BRUCK, Claudine et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

17 April 1999 (17.04.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer P. Regis
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

2640926

ENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference FB/SD/B45110	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/EP 98/ 06040	International filing date *(day/month/year)* 17/09/1998	(Earliest) Priority Date *(day/month/year)* 26/09/1997
Applicant SMITHKLINE BEECHAM BIOLOGICALS S.A. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [X] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [ ] the text is approved as submitted by the applicant
   - [X] the text has been established by this Authority to read as follows:

   FUSION PROTEINS COMPRISING HIV-1 TAT AND7OR NEF PROTEINS

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. –
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/06040

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/49 C12N15/62 C07K14/16 A61K39/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 04686 A (BARSOUM JAMES G ;BIOGEN INC (US); FAWELL STEPHEN E (US); PEPINSKY) 3 March 1994 see page 54 - page 73 ---	1,4, 13-15
X	BODÉUS M ET AL.: "In vitro binding and phosphorylation of human immunodefciency virus type 1 Nef protein by serine/threonine protein kinase" JOURNAL OF GENERAL VIROLOGY, vol. 76, no. 6, June 1995, pages 1337-1344, XP002092508 READING GB see page 1338, left-hand column, paragraph 3 --- -/--	1,5, 13-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 February 1999	18/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SALFELD J ET AL: "A tripartite HIV-1 tat-env-rev fusion protein" EMBO JOURNAL, vol. 9, no. 3, 1 March 1990, pages 965-970, XP000113784 see the whole document	1,4
X	AHMED A AZAD ET AL: "Large-scale production and characterization of recombinant human immunodeficiency virus type 1 Nef" JOURNAL OF GENERAL VIROLOGY, vol. 75, no. 3, 1 March 1994, pages 651-655, XP000565729 see the whole document	1,5, 13-15
A	JANSON H ET AL.: "Protein D, the immunoglobulin D-binding protein of Haemophilus influenzae, is a lipoprotein" INFECTION AND IMMUNITY, vol. 60, no. 4, April 1992, pages 1336-1342, XP002092509 WASHINGTON US cited in the application see the whole document	6-8

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 98/06040

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9404686 A	03-03-1994	AT 173016 T	15-11-1998
		AU 667244 B	14-03-1996
		AU 5083293 A	15-03-1994
		CA 2135642 A	03-03-1994
		DE 69321962 D	10-12-1998
		DE 656950 T	14-03-1996
		EP 0656950 A	14-06-1995
		ES 2123062 T	01-01-1999
		FI 945248 A	05-01-1995
		JP 10033186 A	10-02-1998
		JP 2702285 B	21-01-1998
		JP 7503617 T	20-04-1995
		NO 944273 A	17-02-1995
		NZ 255831 A	24-04-1997
		US 5674980 A	07-10-1997
		US 5670617 A	23-09-1997
		US 5652122 A	29-07-1997
		US 5747641 A	05-05-1998

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
FB/SD/B45110		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP98/06040	17/09/1998	26/09/1997

International Patent Classification (IPC) or national classification and IPC
C12N15/49

Applicant
SMITHKLINE BEECHAM BIOLOGICALS S.A. et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which hav been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
17/04/1999	21.12.99

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
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Authorized officer
Stolz, B

Telephone No. +49 89 2399 8416

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP98/06040

I. Basis of th r port

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-26	as originally filed				

Claims, No.:

1-31	as received on	08/12/1999	with letter of	06/12/1999	

Drawings, sheets:

1-9	as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-31
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-24, 27-31
	No:	Claims	25, 26
Industrial applicability (IA)	Yes:	Claims	1-31
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

1. Reasoned statement

1.1. The application describes fusion proteins consisting of a fragment of H. influenzae protein D and HIV Tat and/or HIV Nef proteins. The molecules produce immune responses in mice and inhibit T-cell activation in vitro.

1.2. Amended claims (Art. 34(2)(b) PCT)

The IPEA does not find a clear basis in the application documents as originally filed leading directly and unambiguously to the subject matter of claims 13 and 18. While there is an example specifying carboxymethylation of a Nef-Tat fusion protein, there seems to be no basis for the carboxymethylation to be applied to all the vaccines. Equally, there are references to oil in water emulsions on p. 8 and p. 23 of the application documents, but they are more specific in that they mention the presence of tocopherol and squalene. Strictly speaking, there is no basis for merely water in oil.

1.3. Novelty (Art. 33(2) PCT)

The new set of claims refers to vaccine compositions comprising HIV Tat and/or Nef fusion proteins (claims 1-19), Nef-Tat or Tat-Nef fusion proteins (claims 20-24), methods of preparing Nef or Tat in P. pastoris (claims 25-29), and a vaccine comprising recombinant Tat-containing protein (claims 30-31). All these objects have not been mentioned in the cited prior art and are therefore novel.

1.4. Inventive step (Art. 33(3) PCT)

Tat-Nef or Nef-Tat fusion proteins as well as the use of Nef or Tat proteins fused to additional proteins for vaccination are not mentioned in the prior art. Therefore, the subject matter of claims 1 to 24, and 27 to 31 cannot be derived from the cited prior art in an obvious way.

Claims 25 and 26 are considered to lack inventive step. HIV Nef and Tat proteins have been known and expressed (see the documents cited in the ISR). P. pastoris is a commonly used host for recombinant protein expression which presents an

obvious alternative to other frequently used hosts. Unless the applicant demonstrates unexpected properties of the expressed protein (when compared to the proteins of the prior art) or the non-obvious solution of problems encountered when expressing the protein in P. pastoris, claims 25 and 26 are considered to lack inventive step.

2. Certain observations

2.1. The terms "Tat" and "Nef" are apparently not used to designate the full-length proteins only (see claims 1 and 9 or 10). Thus, they are used in an arbitrarily defined way and render the scope of the claims unclear.

2.2. Several claims refer to "derivatives". This term is open to interpretation and as such renders the scope of the respective claims unclear.

2.3. The term "fusion partner" is only supported as far as protein-protein fusions are concerned.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/49, 15/62, C07K 14/16, A61K 39/21 — A1

(11) International Publication Number: **WO 99/16884**

(43) International Publication Date: 8 April 1999 (08.04.99)

(21) International Application Number: PCT/EP98/06040

(22) International Filing Date: 17 September 1998 (17.09.98)

(30) Priority Data:
9720585.0 26 September 1997 (26.09.97) GB

(71) Applicant *(for all designated States except US)*: SMITHKLINE BEECHAM BIOLOGICALS S.A. [BE/BE]; Rue de l'Institut 89, B–1330 Rixensart (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BRUCK, Claudine [BE/BE]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B–1330 Rixensart (BE). GODART, Stephane, Andre, Georges [BE/BE]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B–1330 Rixensart (BE). MARCHAND, Martine [BE/BE]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B–1330 Rixensart (BE).

(74) Agent: TYRRELL, Arthur, William, Russell; SmithKline Beecham, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: FUSION PROTEINS COMPRISING HIV–1 TAT AND/OR NEF PROTEINS

(57) Abstract

The invention provides (a) an HIV Tat protein or derivative thereof linked to either (i) a fusion partner or (ii) an HIV Nef protein or derivative thereof; or (b) an HIV Nef protein or derivative thereof linked to either (i) a fusion partner or (ii) an HIV Tat protein or derivative thereof; or (c) an HIV Nef protein or derivative thereof linked to an HIV Tat protein or derivative thereof and a fusion partner. The invention further provides for a nucleic acid encoding such a protein and a host cell, such as Pichia Pastoris, transformed with the aforementioned nucleic acid.

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FUSION PROTEINS COMPRISING HIV-1 TAT AND/OR NEF PROTEINS

The present invention relates to novel HIV protein constructs, to their use in medicine, to pharmaceutical compositions containing them and to methods of their manufacture.

In particular, the invention relates to fusion proteins comprising HIV-1 Tat and/or Nef proteins.

HIV-1 is the primary cause of the acquired immune deficiency syndrome (AIDS) which is regarded as one of the world's major health problems. Although extensive research throughout the world, has been conducted to produce a vaccine, such efforts thus far, have not been successful.

Non-envelope proteins of HIV-1 have been described and include for example internal structural proteins such as the products of the *gag* and *pol* genes and, other non-structural proteins such as Rev, Nef, Vif and Tat (Greene et al., New England J. Med, 324, 5, 308 et seq (1991) and Bryant et al. (Ed. Pizzo), Pediatr. Infect. Dis. J., 11, 5, 390 et seq (1992).

HIV Nef and Tat proteins are early proteins, that is, they are expressed early in infection and in the absence of structural proteins.

According to the present invention there is provided a protein comprising

(a) an HIV Nef protein or derivative thereof linked to either (i) a fusion partner or (ii) an HIV Tat protein or derivative thereof; or

(b) an HIV Tat protein or derivative thereof linked to either (i) a fusion partner or (ii) an HIV Nef protein or derivative thereof; or

(c) an HIV Nef protein or derivative thereof linked to an HIV Tat protein or derivative thereof and a fusion partner.

By 'fusion partner' is meant any protein sequence that is not Tat or Nef. Preferably the fusion partner is protein D or its' lipidated derivative Lipoprotein D, from Haemophilius influenzae B. In particular, it is preferred that the N-terminal

third, i.e. approximately the first 100-130 amino acids are utilised. This is represented herein as Lipo D 1/3. In a preferred embodiment of the invention the Nef protein or derivative thereof may be linked to the Tat protein or derivative thereof. Such Nef-Tat fusions may optionally also be linked to an fusion partner, such as protein D.

The fusion partner is normally linked to the N-terminus of the Nef or Tat protein.

Derivatives encompassed within the present invention include molecules with a C terminal Histidine tail which preferably comprises between 5-10 Histidine residues. Generally, a histidine tail containing n residues is represented herein as His (n). The presence of an histidine (or 'His') tail aids purification. More specifically, the invention provides proteins with the following structure

Lipo D 1/3	-	Nef	-	His $(_6)$
Lipo D 1/3	-	Nef-Tat	-	His $(_6)$
Prot D 1/3	-	Nef	-	His $(_6)$
Prot D 1/3	-	Nef-Tat	-	His $(_6)$
		Nef-Tat	-	His $(_6)$

Figure 1 provides the amino-acid (Seq. ID. No. 7) and DNA sequence (Seq. ID. No. 6) of the fusion partner for such constructs.

In a preferred embodiment the proteins are expressed with a Histidine tail comprising between 5 to 10 and preferably six Histidine residues. These are advantageous in aiding purification. Separate expression, in yeast (Saccharomyces cerevisiae), of Nef (Macreadie I.G. et al., 1993, Yeast 9 (6) 565-573) and Tat (Braddock M et al., 1989, Cell 58 (2) 269-79) has already been reported. Nef protein only is myristilated. The present invention provides for the first time the expression of Nef and Tat separately

in a Pichia expression system (Nef-His and Tat-His constructs), and the successful expression of a fusion construct Nef-Tat-His. The DNA and amino acid sequences of representative Nef-His (Seq. ID. No.s 8 and 9), Tat-His (Seq. ID. No.s 10 and 11)and of Nef-Tat-His fusion proteins (Seq. ID. No.s 12 and 13)are set forth in Figure 2.

Derivatives encompassed within the present invention also include mutated proteins. The term 'mutated' is used herein to mean a molecule which has undergone deletion, addition or substitution of one or more amino acids using well known techniques for site directed mutagenesis or any other conventional method.

A mutated Tat is illustrated in Figure 2 (Seq. ID. No.s 22 and 23) as is a Nef-Tat Mutant-His (Seq. ID. No.s 24 and 25).

The present invention also provides a DNA encoding the proteins of the present invention. Such sequences can be inserted into a suitable expression vector and expressed in a suitable host.

A DNA sequence encoding the proteins of the present invention can be synthesized using standard DNA synthesis techniques, such as by enzymatic ligation as described by D.M. Roberts *et al.* in Biochemistry 1985, *24*, 5090-5098, by chemical synthesis, by *in vitro* enzymatic polymerization, or by PCR technology utilising for example a heat stable polymerase, or by a combination of these techniques.

Enzymatic polymerisation of DNA may be carried out *in vitro* using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50µl or less. Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer, such as 0.05M Tris (pH 7.4), 0.01M $MgCl_2$, 0.01M dithiothreitol, 1mM spermidine, 1mM ATP and 0.1mg/ml bovine serum albumin, at a temperature of 4°C to ambient, generally in a volume of 50ml or less. The chemical synthesis of the DNA polymer or fragments may be carried out by conventional

phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982), or in other scientific publications, for example M.J. Gait, H.W.D. Matthes, M. Singh, B.S. Sproat, and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B.S. Sproat, and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H. Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams *et al.*, Journal of the American Chemical Society, 1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus, and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes *et al.*, EMBO Journal, 1984, 3, 801.

The invention also provides a process for preparing a protein of the invention, the process comprising the steps of :

i) preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes the protein or a derivative thereof

ii) transforming a host cell with said vector

iii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said protein; and

iv) recovering said protein

The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis *et al.*, Molecular Cloning - A Laboratory Manual; Cold Spring Harbor, 1982-1989.

The term 'transforming' is used herein to mean the introduction of foreign DNA into a host cell. This can be achieved for example by transformation, transfection or

infection with an appropriate plasmid or viral vector using e.g. conventional techniques as described in Genetic Engineering; Eds. S.M. Kingsman and A.J. Kingsman; Blackwell Scientific Publications; Oxford, England, 1988. The term 'transformed' or 'transformant' will hereafter apply to the resulting host cell containing and expressing the foreign gene of interest.

The expression vectors are novel and also form part of the invention.

The replicable expression vectors may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment encode the desired product, such as the DNA polymer encoding the protein of the invention, or derivative thereof, under ligating conditions.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic or eukaryotic but preferably is *E. coli* or yeast. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses.

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis *et al.* cited above.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis *et al.* cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as E. coli may be treated with a solution of $CaCl_2$ (Cohen *et al.*, Proc. Nat. Acad. Sci., 1973, *69*, 2110) or with a solution comprising a mixture of RbC1, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbC1 and glycerol. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells. The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis *et al.* and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 50°C.

The product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial, such as E. coli - or yeast such as Pichia; it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Conventional protein isolation techniques include selective precipitation, adsorption chromatography, and affinity chromatography including a monoclonal antibody affinity column.

For proteins of the present invention provided with Histidine tails, purification can easily be achieved by the use of a metal ion affinity column. In a preferred embodiment, the protein is further purified by subjecting it to cation ion exchange chromatography and/or Gel filtration chromatography. The protein is then sterilised by passing through a 0.22 μm membrane.

The proteins of the invention can then be formulated as a vaccine, or the Histidine residues enzymatically cleared.

The proteins of the present invention are provided preferably at least 80% pure more preferably 90% pure as visualised by SDS PAGE. Preferably the proteins appear as a single band by SDS PAGE.

The present invention also provides pharmaceutical composition comprising a protein of the present invention in a pharmaceutically acceptable excipient.

Vaccine preparation is generally described in **New Trends and Developments in Vaccines**, Voller *et al.* (eds.), University Park Press, Baltimore, Maryland, 1978. Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The proteins of the present invention are preferably adjuvanted in the vaccine formulation of the invention. Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

In the formulation of the inventions it is preferred that the adjuvant composition induces a preferential TH1 response. Suitable adjuvant systems include, for example, a combination of monophosphoryl lipid A or derivative thereof, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt.

An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D- MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739.

A particularly potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is a preferred formulation.

Accordingly in one embodiment of the present invention there is provided a vaccine comprising a protein according to the invention adjuvanted with a monophosphoryl lipid A or derivative thereof, especially 3D-MPL.

Preferably the vaccine additionally comprises a saponin, more preferably QS21.

Preferably the formulation additional comprises an oil in water emulsion and tocopherol. The present invention also provides a method for producing a vaccine formulation comprising mixing a protein of the present invention together with a pharmaceutically acceptable excipient, such as 3D-MPL.

The vaccine of the present invention may additional comprise further HIV proteins, such as the envelope glycoprotein gp160 or its derivative gp 120.

In another aspect, the invention relates to an HIV Nef or an HIV Tat protein or derivative thereof expressed in *Pichia pastoris*.

The invention will be further described by reference to the following examples:

EXAMPLES:

General

Nef and Tat proteins, two regulatory proteins encoded by the human immunodeficiency virus (HIV-1) were produced in *E.coli* and in the methylotrophic yeast *Pichia pastoris*.

The *nef* gene from the Bru/Lai isolate (Cell 40: 9-17, 1985) was selected for these constructs since this gene is among those that are most closely related to the consensus Nef .

The starting material for the Bru/Lai *nef* gene was a 1170bp DNA fragment cloned on the mammalian expression vector pcDNA3 (pcDNA3/*nef*).

The *tat* gene originates from the BH10 molecular clone. This gene was received as an HTLV III cDNA clone named pCV1 and described in Science, 229, p69-73, 1985.

1. EXPRESSION OF HIV-1 *nef* AND *tat* SEQUENCES IN E.COLI.

Sequences encoding the Nef protein as well as a fusion of *nef* and *tat* sequences were placed in plasmids vectors: pRIT14586 and pRIT14589 (see figure 1).

Nef and the Nef-Tat fusion were produced as fusion proteins using as fusion partner a part of the protein D. Protein D is an immunoglobulin D binding protein exposed at the surface of the gram-negative bacterium *Haemophilus influenzae*.

pRIT14586 contains, under the control of a λPL promoter, a DNA sequence derived from the bacterium *Haemophilus influenzae* which codes for the first 127 amino acids of the protein D (Infect. Immun. 60 : 1336-1342, 1992), immediately followed by a multiple cloning site region plus a DNA sequence coding for one glycine, 6 histidines residues and a stop codon (Fig. 1A).

This vector is designed to express a processed lipidated His tailed fusion protein (LipoD fusion protein). The fusion protein is synthesised as a precursor with an 18 amino acid resïdues long signal sequence and after processing, the cysteine at position 19 in the precursor molecule becomes the amino terminal residue which is then modified by covalently bound fatty acids (Fig.1B).

pRIT14589 is almost identical to pRIT14586 except that the protD derived sequence starts immediately after the cysteine19 codon.
Expression from this vector results in a His tailed, non lipidated fusion protein (Prot D fusion protein).

Four constructs were made: LipoD-*nef*-His, LipoD-*nef-tat*-His, ProtD-*nef*-His, and ProtD-*nef-tat*-His.

The first two constructs were made using the expression vector pRIT14586, the last two constructs used pRIT14589.

1.1 CONSTRUCTION OF THE RECOMBINANT STRAIN ECLD-N1 PRODUCING THE LIPOD-Nef-HIS FUSION PROTEIN.

1.1.1 Construction of the lipoD-*nef*-His expression plasmid pRIT14595

The *nef* gene(Bru/Lai isolate) was amplified by PCR from pcDNA3/Nef plasmid with primers 01 and 02.

NcoI

PRIMER 01 (Seq ID NO 1): 5'ATCGTCCATG.GGT.GGC.AAG.TGG.T 3'

SpeI

PRIMER 02 (Seq ID NO 2): 5' CGGCTACTAGTGCAGTTCTTGAA 3'

The *nef* DNA region amplified starts at nucleotide 8357 and terminates at nucleotide 8971 (Cell, 40: 9-17, 1985).

An NcoI restriction site (which carries the ATG codon of the *nef* gene) was introduced at the 5'end of the PCR fragment while a SpeI site was introduced at the 3' end.

The PCR fragment obtained and the expression plasmid pRIT14586 were both restricted by NcoI and SpeI, purified on an agarose gel, ligated and transformed in the

appropriate *E.coli* host cell, strain AR58.This strain is a cryptic λ lysogen derived from N99 that is *gal*E::Tn10, Δ-8 (*chl*D-*pgl*), Δ-H1 (*cro-chl*A), N^+, and cI857.

The resulting recombinant plasmid received, after verification of the *nef* amplified region by automatic sequencing,(see section 1.1.2 below) the pRIT14595 denomination.

1.1.2 Selection of transformants of *E. Coli* strain AR58 with pRIT14595

When transformed in AR58 *E.coli* host strain, the recombinant plasmid directs the heat-inducible production of the heterologous protein.

Heat inducible protein production of several recombinant lipoD-Nef-His transformants was analysed by Coomassie Blue stained SDS-PAGE. All the transformants analysed showed an heat inducible heterologous protein production. The abundance of the recombinant Lipo D-Nef-Tat-His fusion protein was estimated at 10% of total protein.

One of the transformants was selected and given the laboratory accession number ECLD-N1.

The recombinant plasmid was reisolated from strain ECLD-N1, and the sequence of the *nef*-His coding region was confirmed by automated sequencing .This plasmid received the official designation pRIT14595.

The fully processed and acylated recombinant Lipo D-*nef*-His fusion protein produced by strain ECLD-N1 is composed of:

- °Fatty acids
- °109 a.a. of proteinD (starting at a.a.19 and extending to a.a.127).

°A methionine, created by the use of NcoI cloning site of pRIT14586 (Fig.1).

°205a.a. of Nef protein (starting at a.a.2 and extending to a.a.206).

°A threonine and a serine created by the cloning procedure (cloning at SpeI site of pRIT14586).

°One glycine and six histidines.

1.2 CONSTRUCTION OF RECOMBINANT STRAIN ECD-N1 PRODUCING PROT D-Nef-HIS FUSION PROTEIN.

Construction of expression plasmid pRIT14600 encoding the Prot D-Nef-His fusion protein was identical to the plasmid construction described in example 1.1.1 with the exception that pRIT14589 was used as receptor plasmid for the PCR amplified *nef* fragment.

E.coli AR58 strain was transformed with pRIT14600 and transformants were analysed as described in example 1.1.2. The transformant selected received laboratory accession number ECD-N1.

1.3 CONSTRUCTION OF RECOMBINANT STRAIN ECLD-NT6 PRODUCING THE LIPO D-Nef-Tat-HIS FUSION PROTEIN.

1.3.1 Construction of the lipo D-Nef-Tat-His expression plasmid pRIT14596

The *tat* gene(BH10 isolate) was amplified by PCR from a derivative of the pCV1 plasmid with primers 03 and 04. SpeI restriction sites were introduced at both ends of the PCR fragment.

SpeI

PRIMER 03 (Seq ID NO 3): 5' ATCGTACTAGT.GAG.CCA.GTA.GAT.C 3'

SpeI

PRIMER 04 (Seq ID NO 4): 5' CGGCTACTAGTTTCCTTCGGGCCT 3'

The nucleotide sequence of the amplified *tat* gene is illustrated in the pCV1 clone (Science 229 : 69-73, 1985) and covers nucleotide 5414 till nucleotide 7998.

The PCR fragment obtained and the plasmid pRIT14595 (expressing lipoD-Nef-His protein) were both digested by SpeI restriction enzyme, purified on an agarose gel, ligated and transformed in competent AR58 cells. The resulting recombinant plasmid received, after verification of the *tat* amplified sequence by automatic sequencing (see section 1.3.2 below), the pRIT14596 denomination.

1.3.2 Selection of transformants of strain AR58 with pRIT14596

Transformants were grown, heat induced and their proteins were analysed by Coomassie Blue stained gels. The production level of the recombinant protein was estimated at 1% of total protein. One recombinant strain was selected and received the laboratory denomination ECLD-NT6.

The lipoD-*nef- tat* -His recombinant plasmid was reisolated from ECLD-NT6 strain, sequenced and received the official designation pRIT14596.

The fully processed and acylated recombinant Lipo D-Nef-Tat-His fusion protein produced by strain ECLD-N6 is composed of:

°Fatty acids
°109 a.a. of proteinD (starting at a.a.19 and extending to a.a.127).
°A methionine, created by the use of NcoI cloning site of pRIT14586.
°205a.a. of the Nef protein (starting at a.a.2 and extending to a.a.206)
°A threonine and a serine created by the cloning procedure
°85a.a. of the Tat protein (starting at a.a.2 and extending to a.a.86)
°A threonine and a serine introduced by cloning procedure
°One glycine and six histidines.

1.4 CONSTRUCTION OF RECOMBINANT STRAIN ECD-NT1 PRODUCING PROT D-Nef-Tat-HIS FUSION PROTEIN.

Construction of expression plasmid pRIT14601 encoding the Prot D-Nef-Tat-His fusion protein was identical to the plasmid construction described in example 1.3.1 with the exception that pRIT14600 was used as receptor plasmid for the PCR amplified *nef* fragment.

E.coli AR58 strain was transformed with pRIT14601 and transformants were analysed as described previously. The transformant selected received laboratory accession number ECD-NT1.

2. EXPRESSION OF HIV-1 *nef* AND *tat* SEQUENCES IN PICHIA PASTORIS.

Nef protein, Tat protein and the fusion Nef -Tat were expressed in the methylotrophic yeast *Pichia pastoris* under the control of the inducible alcohol oxidase (AOX1) promoter.

To express these HIV-1 genes a modified version of the integrative vector PHIL-D2 (INVITROGEN) was used. This vector was modified in such a way that expression of heterologous protein starts immediately after the native ATG codon of the AOX1 gene and will produce recombinant protein with a tail of one glycine and six histidines residues . This PHIL-D2-MOD vector was constructed by cloning an oligonucleotide linker between the adjacent AsuII and EcoRI sites of PHIL-D2 vector (see Figure 3). In addition to the His tail, this linker carries NcoI, SpeI and XbaI restriction sites between which *nef*, *tat* and *nef-tat* fusion were inserted.

2.1 CONSTRUCTION OF THE INTEGRATIVE VECTORS pRIT14597 (encoding Nef-His protein), pRIT14598 (encoding Tat-His protein) and pRIT14599 (encoding fusion Nef-Tat-His).

The *nef* gene was amplified by PCR from the pcDNA3/Nef plasmid with primers 01 and 02(see section 1.1.1 construction of pRIT14595).The PCR fragment obtained and the integrative PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14597 (see Figure 3).

The *tat* gene was amplified by PCR from a derivative of the pCV1 plasmid with primers 05 and 04(see section 1.3.1 construction of pRIT14596):

NcoI

PRIMER 05 (Seq ID NO 5): 5'ATCGTCCATGGAGCCAGTAGATC 3'

An NcoI restriction site was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end with primer 04. The PCR fragment obtained and the PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14598.

To construct pRIT14599, a 910bp DNA fragment corresponding to the *nef-tat*-His coding sequence was ligated between the EcoRI blunted(T4 polymerase) and NcoI sites of the PHIL-D2-MOD vector. The *nef-tat*-His coding fragment was obtained by XbaI blunted(T4 polymerase) and NcoI digestions of pRIT14596.

2.2 TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115(his4).

To obtain *Pichia pastoris* strains expressing Nef-His, Tat-His and the fusion Nef-Tat-His, strain GS115 was transformed with linear NotI fragments carrying the respective expression cassettes plus the HIS4 gene to complement his4 in the host genome.Transformation of GS115 with NotI-linear fragments favors recombination at the AOXI locus.

Multicopy integrant clones were selected by quantitative dot blot analysis and the type of integration, insertion (Mut^+phenotype) or transplacement (Mut^sphenotype), was determined.

From each transformation, one transformant showing a high production level for the recombinant protein was selected :

Strain Y1738 (Mut^+ phenotype) producing the recombinant Nef-His protein, a myristylated 215 amino acids protein which is composed of:

- °Myristic acid
- °A methionine, created by the use of NcoI cloning site of PHIL-D2-MOD vector
- °205 a.a. of Nef protein(starting at a.a.2 and extending to a.a.206)

°A threonine and a serine created by the cloning procedure (cloning at SpeI site of PHIL-D2-MOD vector.

°One glycine and six histidines.

Strain Y1739 (Mut^+ phenotype) producing the Tat-His protein, a 95 amino acid protein which is composed of:

°A methionine created by the use of NcoI cloning site

°85 a.a. of the Tat protein(starting at a.a.2 and extending to a.a.86)

°A threonine and a serine introduced by cloning procedure

°One glycine and six histidines

Strain Y1737(Mut^s phenotype) producing the recombinant Nef-Tat-His fusion protein, a myristylated 302 amino acids protein which is composed of:

°Myristic acid

°A methionine, created by the use of NcoI cloning site

°205a.a. of Nef protein(starting at a.a.2 and extending to a.a.206)

°A threonine and a serine created by the cloning procedure

°85a.a. of the Tat protein(starting at a.a.2 and extending to a.a.86)

°A threonine and a serine introduced by the cloning procedure

°One glycine and six histidines

3. EXPRESSION OF HIV-1 Tat-MUTANT IN PICHIA PASTORIS

As well as a Nef-Tat mutant fusion protein, a mutant recombinant Tat protein has also been expressed. The mutant Tat protein must be **biologically inactive** while **maintaining** its **immunogenic epitopes.**

A double mutant *tat* gene, constructed by D.Clements (Tulane University) was selected for these constructs.

This *tat* gene (originates from BH10 molecular clone) bears **mutations** in the **active site region (Lys41→Ala)**and in **RGD motif (Arg78→Lys and Asp80→Glu)** (Virology 235: 48-64, 1997).

The mutant *tat* gene was received as a cDNA fragment subcloned between the EcoRI and HindIII sites within a CMV expression plasmid (pCMVLys41/KGE)

3.1 CONSTRUCTION OF THE INTEGRATIVE VECTORS pRIT14912(encoding Tat mutant-His protein) and pRIT14913(encoding fusion Nef-Tat mutant-His).

The *tat* mutant gene was amplified by PCR from the pCMVLys41/KGE plasmid with primers 05 and 04 (see section 2.1construction of pRIT14598)

An NcoI restriction site was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end with primer 04. The PCR fragment obtained and the PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14912

To construct pRIT14913, the *tat* mutant gene was amplified by PCR from the pCMVLys41/KGE plasmid with primers 03 and 04 (see section 1.3.1 construction of pRIT14596).

The PCR fragment obtained and the plasmid pRIT14597 (expressing Nef-His protein) were both digested by SpeI restriction enzyme, purified on agarose gel and ligated to create the integrative plasmid pRIT14913

3.2 TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115.

Pichia pastoris strains expressing Tat mutant-His protein and the fusion Nef-Tat mutant-His were obtained, by applying integration and recombinant strain selection strategies previously described in section 2.2 .

Two recombinant strains producing Tat mutant-His protein ,a 95 amino-acids protein, were selected: Y1775 (Mut^+ phenotype) and Y1776(Mut^s phenotype).

One recombinant strain expressing Nef-Tat mutant-His fusion protein, a 302 amino-acids protein was selected: Y1774(Mut^+ phenotype).

4. PURIFICATION OF Nef-Tat-His FUSION PROTEIN (PICHIA PASTORIS)

The purification scheme has been developed from 146g of recombinant Pichia pastoris cells (wet weight) or 2L Dyno-mill homogenate OD 55. The chromatographic steps are performed at room temperature. Between steps , Nef-Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

146g of Pichia pastoris cells

↓

Homogenization — Buffer: 2L 50 mM PO_4 pH 7.0, final OD:50

↓

Dyno-mill disruption (4 passes)

↓

Centrifugation — JA10 rotor / 9500 rpm/ 30 min / room temperature

↓

Dyno-mill Pellet

↓

Wash (1h - 4°C) — Buffer: +2L 10 mM PO_4 pH 7.5 - 150mM - NaCl 0,5% empigen

↓

Centrifugation — JA10 rotor / 9500 rpm/ 30 min / room temperature

↓

Pellet

↓

Solubilisation (O/N - 4°C)	Buffer: + 660ml 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl
↓	
Reduction (4H - room temperature - in the dark)	+ 0,2M 2-mercaptoethanesulfonic acid, sodium salt (powder addition) / pH adjusted to 7.5 (with 0,5M NaOH solution) before incubation
↓	
Carboxymethylation (1/2 h - room temperature - in the dark)	+ 0,25M Iodoacetamid (powder addition) / pH adjusted to 7.5 (with 0,5M NaOH solution) before incubation
↓	
Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 30 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl Washing buffer: 1) Equilibration buffer 7.5 - 150mM 2) 10 mM PO_4 pH NaCl - 6M Urea 7.5 - 150mM mM 3) 10 mM PO_4 pH NaCl - 6M Urea - 25 Imidazol Elution buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea - 0,5M Imidazol
↓	
Dilution	Down to an ionic strength of 18 mS/cm² Dilution buffer: 10 mM PO_4 pH 7.5 - 6M Urea
↓	
Cation exchange chromatography on SP Sepharose FF (Pharmacia - 30 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 6.0M Urea

	Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 - 250mM NaCl - 6M Urea Elution buffer: 10 mM Borate pH 9.0 - 2M NaCl - 6M Urea
↓	
Concentration	up to 5 mg/ml 10kDa Omega membrane(Filtron)
↓	
Gel filtration chromatography on Superdex200 XK 16/60 (Pharmacia - 120 ml of resin)	Elution buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea 5 ml of sample / injection → 5 injections
↓	
Dialysis (O/N - 4°C)	Buffer: 10 mM PO_4 pH 6.8 - 150mM NaCl - 0,5M Arginin*
↓	
Sterile filtration	Millex GV 0,22µm

* ratio: 0,5M Arginin for a protein concentration of 1600µg/ml.

Purity

The level of purity as estimated by SDS-PAGE is shown in Figure 4 by Daiichi Silver Staining and in Figure 5 by Coomassie blue G250.

After Superdex200 step: > 95%

After dialysis and sterile filtration steps: > 95%

Recovery

51mg of Nef-Tat-his protein are purified from 146g of recombinant Pichia pastoris cells (= 2L of Dyno-mill homogenate OD 55)

5. VACCINE PREPARATION

A vaccine prepared in accordance with the invention comprises the expression product of a DNA recombinant encoding an antigen as exemplified in example 1 or 2 and as adjuvant, the formulation comprising a mixture of 3 de -O-acylated monophosphoryl lipid A 3D-MPL and QS21 in an oil/water emulsion.

3D-MPL: is a chemically detoxified form of the lipopolysaccharide (LPS) of the Gram-negative bacteria Salmonella minnesota.

Experiments performed at Smith Kline Beecham Biologicals have shown that 3D-MPL combined with various vehicles strongly enhances both the humoral and a TH1 type of cellular immunity.

QS21: is one saponin purified from a crude extract of the bark of the Quillaja Saponaria Molina tree, which has a strong adjuvant activity: it activates both antigen-specific lymphoproliferation and CTLs to several antigens.
Experiments performed at Smith Kline Beecham Biologicals have demonstrated a clear synergistic effect of combinations of 3D-MPL and QS21 in the induction of both humoral and TH1 type cellular immune responses.

The oil/water emulsion is composed of 2 oils (a tocopherol and squalene), and of PBS containing Tween 80 as emulsifier. The emulsion comprised 5% squalene 5%

tocopherol 0.4% Tween 80 and had an average particle size of 180 nm (see WO 95/17210).

Experiments performed at Smith Kline Beecham Biologicals have proven that the adjunction of this O/W emulsion to 3D-MPL/QS21 further increases their immunostimulant properties.

Preparation of the oil/water emulsion (2 fold concentrate)

Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100ml two fold concentrate emulsion 5g of DL alpha tocopherol and 5ml of squalene are vortexed to mix thoroughly. 90ml of PBS/Tween solution is added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M110S microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

Preparation of oil in water formulation.

Antigen prepared in accordance with example 1 or 2 (5µg) was diluted in 10 fold concentrated PBS pH 6.8 and H_2O before consecutive addition of SB62, 3D-MPL (5µg), QS21 (5µg) and 50 µg/ml thiomersal as preservative at 5 min interval. The emulsion volume is equal to 50% of the total volume (50µl for a dose of 100µl).

All incubations were carried out at room temperature with agitation.

6. IMMUNOGENICITY OF Tat AND Nef-Tat IN RODENTS

Characterization of the immune response induced after immunization with Tat and NefTat was carried out. To obtain information on isotype profiles and cell-mediated immunity (CMI) two immunization experiments in mice were conducted. In the first experiment mice were immunized twice two weeks apart into the footpad with Tat or

NefTat in the oxydized or reduced form, respectively. Antigens were formulated in an oil in water emulsion comprising squalene, tween 80 ™ (polyoxyethylene sorbitan monooleate) QS21, 3D-MPL and α-tocopherol, and a control group received the adjuvant alone. Two weeks after the last immunization sera were obtained and subjected to Tat-specific ELISA (using reduced Tat for coating) for the determination of antibody titers and isotypes (Figure 6a). The antibody titers were highest in the mice having received oxydized Tat. In general, the oxydized molecules induced higher antibody titers than the reduced forms, and Tat alone induced higher antibody titers than NefTat. The latter observation was confirmed in the second experiment. Most interestingly, the isotype profile of Tat-specific antibodies differed depending on the antigens used for immunization. Tat alone elicited a balanced IgG1and IgG2a profile, while NefTat induced a much stronger T_{H2} bias (Figure 6b). This was again confirmed in the second experiment.

In the second mouse experiment animals received only the reduced forms of the molecules or the adjuvant alone. Besides serological analysis (see above) lymphoproliferative responses from lymph node cells were evaluated. After restimulation of those cells in vitro with Tat or NefTat ^{3}H-thymidine incorporation was measured after 4 days of culture. Presentation of the results as stimulation indices indicates that very strong responses were induced in both groups of mice having received antigen (Figure 7).

In conclusion, the mice studies indicate that Tat as well as Nef-Tat are highly immunogenic candidate vaccine antigens. The immune response directed against the two molecules is characterized by high antibody responses with at least 50% IgG1. Furthermore, strong CMI responses (as measured by lymphoproliferation) were observed.

7. FUNCTIONAL PROPERTIES OF THE Tat AND Nef-Tat PROTEINS

The Tat and NefTat molecules in oxydized or reduced form were investigated for their ability to bind to human T cell lines. Furthermore, the effect on growth of

those cell lines was assessed. ELISA plates were coated overnight with different concentration of the Tat and NefTat proteins, the irrelevant gD from herpes simplex virus type II, or with a buffer control alone. After removal of the coating solution HUT-78 cells were added to the wells. After two hours of incubation the wells were washed and binding of cells to the bottom of the wells was assessed microscopically. As a quantitative measure cells were stained with toluidine blue, lysed by SDS, and the toluidine blue concentration in the supernatant was determined with an ELISA plate reader. The results indicate that all four proteins, Tat and NefTat in oxydized or reduced form mediated binding of the cells to the ELISA plate (Figure 8). The irrelevant protein (data not shown) and the buffer did not fix the cells. This indicates that the recombinantly expressed Tat-containing proteins bind specifically to human T cell lines.

In a second experiment HUT-78 cells were left in contact with the proteins for 16 hours. At the end of the incubation period the cells were labeled with [^{3}H]-thymidine and the incorporation rate was determined as a measure of cell growth. All four proteins included in this assay inhibited cell growth as judged by diminished radioactivity incorporation (Figure 9). The buffer control did not mediate this effect. These results demonstrate that the recombinant Tat-containing proteins are capable of inhibiting growth of a human T cell line.

In summary the functional characterization of the Tat and NefTat proteins reveals that these proteins are able to bind to human Tcell lines. Furthermore, the proteins are able to inhibit growth of such cell lines.

CLAIMS

1. A protein comprising

(a) an HIV Tat protein or derivative thereof linked to either (i) a fusion partner or (ii) an HIV Nef protein or derivative thereof; or

(b) an HIV Nef protein or derivative thereof linked to either (i) a fusion partner or (ii) an HIV Tat protein or derivative thereof; or

(c) an HIV Nef protein or derivative thereof linked to an HIV Tat protein or derivative thereof and a fusion partner.

2. A protein as claimed in claim 1 which is a Tat-Nef fusion protein or derivative thereof.

3. A protein as claimed in claim 1 which is a Nef-Tat fusion protein or derivative thereof.

4. A protein according to claim 1 wherein the derivative of the Tat protein is a mutated Tat protein.

5. A protein according to claim 1 wherein the derivative of the Nef protein is a mutated Nef protein.

6. A Protein as claimed in any one of claims 1 - 5 wherein the fusion partner is a lipoprotein or derivative thereof.

7. A protein as claimed in claim 6 wherein the lipoprotein is Haemophilus Influenza B protein D or derivative thereof.

8. A protein as claimed in Claim 7 wherein the fusion partner comprises between 100-130 amino acid from the N terminal of Haemophilus Influenza B protein D.

9. A protein as claimed in any one of Claims 1 to 8, wherein the Tat protein is the entire Tat protein.

10. A protein as claimed in any one of Claims 1 to 8, wherein the Nef protein is the entire Nef protein.

11. A protein as claimed in any one of Claims 1 to 10, wherein the Tat protein is fused to an HIV Nef protein and a fusion partner.

12. A protein as claimed in any one of Claims 1 to 11, wherein the protein has a Histidine tail.

13. A nucleic acid encoding a protein of Claims 1 to 12.

14. A host transformed with a nucleic acid of Claim 13.

15. A host as claimed in claim 14 wherein the host is either Pichia pastoris or E. coli.

16. A vaccine comprising a protein of any one of Claims 1 to 12 in admixture with a pharmaceutically acceptable excipient.

17. A vaccine of Claim 16 additionally comprising an adjuvant.

18. A vaccine of claim 17 wherein the adjuvant is a TH1 inducing adjuvant.

19. A vaccine as claimed in Claim 17 or 18 which adjuvant comprises monophosphoryl lipid A or derivative thereof such as 3 de-O-acylated monophosphoryl lipid A.

20. A vaccine as claimed in any one of Claims 16 to 19 additionally comprising a saponin adjuvant.

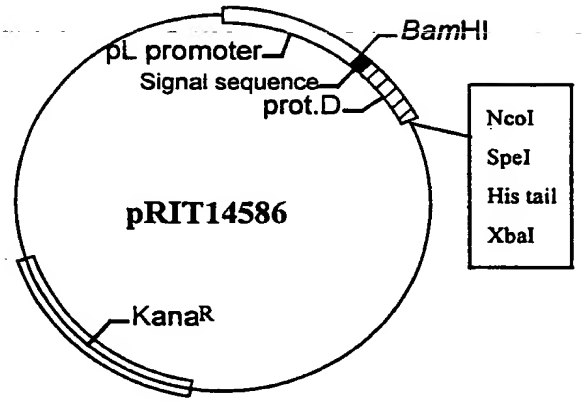
21. A method of producing a protein of Claim 1 to 12, comprising the steps of transforming a host with a nucleic acid encoding said protein, expressing said protein and recovering the protein.

22. A method as claimed in Claim 21 wherein the host is E. coli. or *Pichia pastoris.*

23. A method of producing a vaccine of Claim 16 to 20, comprising admixing the protein of Claim 1 to 12 with a pharmaceutically acceptable diluent.

24. A method of preparing (i) an HIV Nef protein or derivative thereof or (ii) an HIV Tat protein or derivative thereof in *Pichia pastoris* which method comprises the steps of transforming Pichia pastoris with DNA encoding said HIV Nef protein or derivative thereof or HIV Tat protein or derivative thereof, expressing said protein and recovering the protein.

Figure 1: A/ Map of plasmid pRIT14586



B/ Coding sequence of the first 127 amino acids of protein D and multiple cloning site. The signal sequence is underlined.

BamHI
ATG GAT CCA AAA ACT TTA GCC CTT TCT TTA TTA GCA GCT GGC GTA CTA GCA GGT TGT AGC AGC
Met Asp Pro Lys Thr Leu Ala Leu Ser Leu Leu Ala Ala Gly Val Leu Ala Gly Cys Ser Ser
CAT TCA TCA AAT ATG GCG AAT ACC CAA ATG AAA TCA GAC AAA ATC ATT ATT GCT CAC CGT GGT
His Ser Ser Asn Met Ala Asn Thr Gln Met Lys Ser Asp Lys Ile Ile Ile Ala His Arg Gly
GCT AGC GGT TAT TTA CCA GAG CAT ACG TTA GAA TCT AAA GCA CTT GCT TTT GCA CAA CAG GCT
Ala Ser Gly Tyr Leu Pro Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala
GAT TAT TTA GAG CAA GAT TTA GCA ATG ACT AAG GAT GGT CGT TTA GTG GTT ATT CAC GAT CAC
Asp Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val Ile His Asp His
TTT TTA GAT GGC TTG ACT GAT GTT GCG AAA AAA TTC CCA CAT CGT CAT CGT AAA GAT GGC CGT
Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe Pro His Arg His Arg Lys Asp Gly Arg
TAC TAT GTC ATC GAC TTT ACC TTA AAA GAA ATT GAA AGT TTA GAA ATG ACA GAA AAC TTT GAA
Tyr Tyr Val Ile Asp Phe Thr Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu
NcoI SpeI XbaI
ACC ATG GCC ACG TGT GAT CAG AGC TCA ACT AGT GGA CAC CAT CAC CAT CAC CAT TAA TCT AGA
Thr Met Ala Thr Cys Asp Gln Ser Ser Thr Ser Gly His His His His His His *

The amino acid sequence of Figure 1 relates to Seq. ID no. 7 and the nucleic acid sequence of Figure 1 relates to Seq. ID. No. 6.

The DNA and amino acid sequences of Nef-His; Tat-His; Nef-Tat-His fusion and mutated Tat is illustrated.

Pichia-expressed constructs (plain constructs)

⇒ ***Nef - HIS***

DNA sequence (Seq. ID. No. 8)

ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGA
ATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAA
AAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGG
CTAGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTA
AGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGG
GGACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATC
TACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTC
AGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAG
GTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCAT
GGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCA
TTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGC
CACCATCACCATCACCATTAA

Protein sequence(Seq. ID. No. 9)

MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAW
LEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWI
YHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLH
GMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSGHHHHHH.

⇒ ***Tat - HIS***

DNA sequence (Seq. ID. No. 10)

ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAA
ACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTC
ATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGA
CCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAA

TCCCGAGGGGACCCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATCACCAT
TAA

Protein sequence (Seq. ID. No. 11)

MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRR
PPQGSQTHQVSLSKQPTSQSRGDPTGPKETSGHHHHHH.

⇒ ***Nef - Tat - HIS***

DNA sequence (Seq. ID. No. 12)

ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGA
ATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAA
AAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGG
CTAGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTA
AGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGG
GGACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATC
TACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTC
AGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAG
GTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCAT
GGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCA
TTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAG
CCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCT
TGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACA
AAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCT
CAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGA
GGGGACCCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATCACCATTAA

Protein sequence(Seq. ID. No. 13)

^^

MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAW
LEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWI
YHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLH
GMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTA
CTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSR
GDPTGPKETSGHHHHHH.

E.coli-expressed constructs (fusion constructs)

⇒ ***LipoD-Nef-HIS***

DNA sequence (Seq. ID. No. 14)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.
The Lipidation Signal Sequence is underlined. After processing, the cysteine coded by the TGT codon, indicated with a star, becomes the amino terminal residue which is then modified by covalently bound fatty acids.

*ATGGATCCAAAAACTTTAGCCCTTTCTTTATTAGCAGCTGGCGTACTAGCAGGT*TGT
AGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAAATCATTATT
GCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCTAAAGCACTT
GCTTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACTAAGGATGGT
CGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAA
TTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAA
GAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGCAAGTGGTCA
AAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCA
GCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACA
AGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCACAAGAGGAG
GAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAG
GCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATT
CACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTT
GGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAA
GGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGAG
AGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGA
GAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGCCACCATCACCATCACCAT
TAA

Protein sequence of the processed lipidated ProtD-Nef-HIS protein (Seq. ID. No. 15)

(Amino-acids corresponding to Prot D fusion partner are in bold)

CSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMTKD
GRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGGKW
SKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQE
EEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQG
YFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDP
EREVLEWRFDSRLAFHHVARELHPEYFKNCTSGHHHHHH.

⇒ ***LipoD-Nef-Tat-HIS***

DNA sequence (Seq. ID. No. 16)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.
The Lipidation Signal Sequence is underlined. After processing, the cysteine coded by the TGT codon, indicated with a star, becomes the amino terminal residue which is then modified by covalently bound fatty acids.

*

ATGGATCCAAAAACTTTAGCCCTTTCTTTATTAGCAGCTGGCGTACTAGCAGGTTGT
AGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAAATCATTATT
GCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCTAAAGCACTT
GCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACTAAGGATGGT
CGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAA
TTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAA
GAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGCAAGTGGTCA
AAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCA
GCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACA
AGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCACAAGAGGAG
GAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAG
GCAGCTGTAGATCTTAGCCACTTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATT
CACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTT
GGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAA
GGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGAG
AGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGA
GAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCCTAGACTA
GAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCTTGTACCAATTGCTATTGT
AAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCC
TATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCAT
CAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGAGGGGACCCGACAGGCCCG
AAGGAAACTAGTGGCCACCATCACCATCACCATTAA

Protein sequence of the processed lipidated ProtD-NEF-TAT-HIS protein (Seq. ID. No. 17)

(Amino-acids corresponding to Prot D fusion partner are in bold)

CSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMTKD
GRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGGKW
SKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQE
EEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQG
YFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDP
EREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCY
CKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTG
PKETSGHHHHHH.

⇒ ***ProtD-Nef -HIS***

DNA sequence (Seq. ID. No. 18)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAA
ATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCT
AAAGCACTTGCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACT
AAGGATGGTCGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTT
GCGAAAAAATTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTT
ACCTTAAAAGAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGC
AAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGA
GCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGA
GCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCA
CAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATG
ACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAA
GGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACA
CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCA
CTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAG
GCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGAT
GACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCAC
GTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGCCACCATCAC
CATCACCATTAA

Protein sequence (Seq. ID. No. 19)

(Amino-acids corresponding to Prot D fusion partner are in bold)

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYL
EQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLK
EIQSLEMTENFETMGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDL
EKHGAITSSNTAATNAACAWLEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSH
FLKEKGGLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGW
CYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRFDSRLAFH
HVARELHPEYFKNCTSGHHHHHH.

⇒ ***ProtD-Nef -Tat-HIS***

DNA sequence (Seq. ID. No. 20)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAA
ATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCT
AAAGCACTTGCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACT
AAGGATGGTCGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTT
GCGAAAAAATTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTT
ACCTTAAAAGAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGC
AAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGA
GCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGA
GCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCA
CAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATG
ACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAA
GGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACA
CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCA
CTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAG
GCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGAT
GACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCAC
GTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGAT
CCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCTTGTACCAAT
TGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACAAAAGCCTTA
GGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGT
CAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGAGGGGACCCG
ACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATCACCATTAA

Protein sequence (Seq. ID. No. 21)

(Amino-acids corresponding to Prot D fusion partner are in bold)

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMT
KDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGG
KWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEA
QEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHT
QGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMD
DPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTN
CYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDP
TGPKETSGHHHHHH.

⇒ Tat-MUTANT-HIS

DNA sequence (Seq. ID. No. 22)

```
ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATC   40
CAGGAAGTCAGCCTAAAACTGCTTGTACCAATTGCTATTG   80
TAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACA  120
GCTGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGAC  160
AGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGT  200
TTCTCTATCAAAGCAACCCACCTCCCAATCCAAAGGGGAG  240
CCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATC  280
ACCATTAA                                  288
```

Protein sequence(Seq. ID. No. 23)

Mutated amino-acids in Tat sequences are in bold.

```
MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFIT   40
AALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSKGE   80
PTGPKETSGHHHHHH.                           95
```

⇒Nef-Tat-Mutant-HIS

DNA sequence(Seq. ID. No. 24)

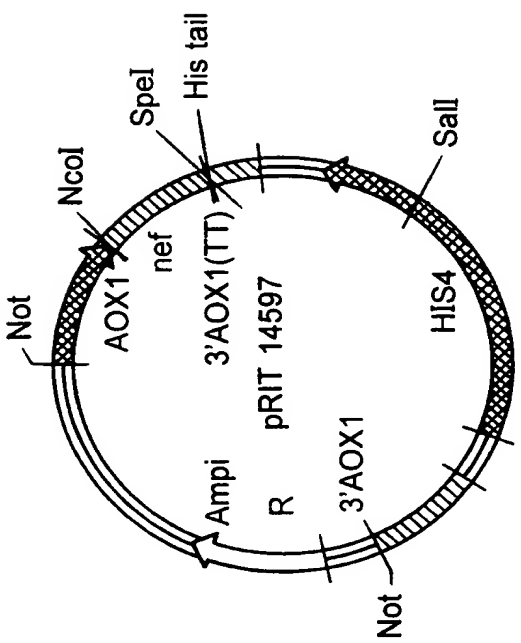
```
ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGC   40
CTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCAGCAGC   80
AGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACAT  120
GGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTG  160
CTTGTGCCTGGCTAGAAGCACAAGAGGAGGAGGAGGTGGG  200
TTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACT  240
TACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAA  280
AGGGGGGACTGGAAGGGCTAATTCACTCCCAACGAAGACA  320
AGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC  360
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCA  400
GATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACC  440
AGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGAGAG  480
AACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGG  520
ATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAG  560
CCGCCTAGCATTTCATCACGTGGCCCGAGAGCTGCATCCG  600
GAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCCTA  640
GACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAAC  680
TGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCAT  720
TGCCAAGTTTGTTTCATAACAGCTGCCTTAGGCATCTCCT  760
ATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCA  800
AGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCC  840
ACCTCCCAATCCAAAGGGGAGCCGACAGGCCCGAAGGAAA  880
CTAGTGGCCACCATCACCATCACCATTAA              909
```

Protein sequence (Seq. ID. No. 25)

Mutated amino-acids in Tat sequence are in bold.

```
MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKH  40
GAITSSNTAATNAACAWLEAQEEEEVGFPVTPQVPLRPMT  80
YKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQGY 120
FPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGE 160
NTSLLHPVSLHGMDDPEREVLEWRFDSRLAFHHVARELHP 200
EYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFH 240
CQVCFITAALGISYGRKKRRQRRRPPQGSQTHQVSLSKQP 280
TSQSKGEPTGPKETSGHHHHHH.                   302
```

Fig . 3 Map of pRIT14597 integrative vector



MCS POLYLINKER: *nef* gene inserted between NcoI and SpeI sites.

Acu II *Nco I* *Spe I* *Eco RI*

TTCGAA.ACC.ATGGCCGCGGACTAGT.GGC.CAC.CAT.CAC.CAT.CAC.CAT.TAA.CGGAATTC

Thr .Ser . Gly. His . His . His . His . His . His

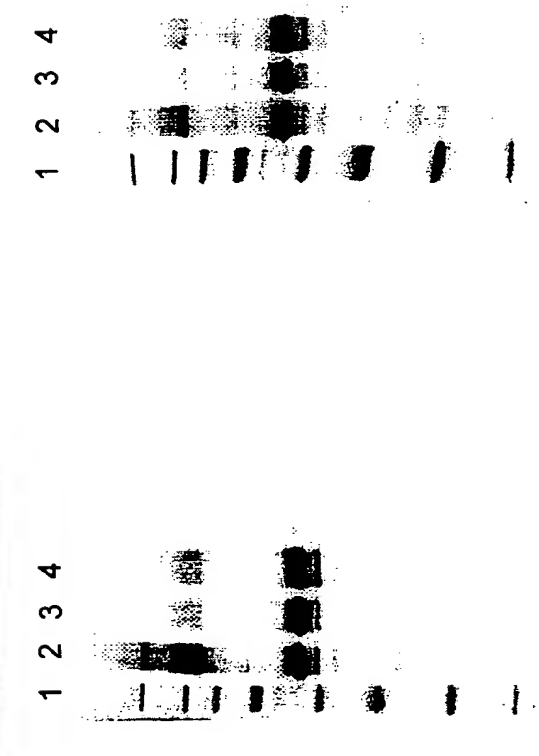
The amino acid sequence of Figure 3 relates to Seq. ID no. 27 and the nucleic acid sequence of Figure 3 relates to Seq. ID. No.26.

Fig. 4 SDS-PAGE: Nef-Tat-his fusion protein

1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa
2: TNH/23 SP eluate (250 ng)
3: TNH/23 Purified bulk (250 ng)
4: TNH/22 Purified bulk (250 ng)

5: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa
6: TNH/23 SP eluate (400 ng)
7: TNH/23 Purified bulk (400 ng)
8: TNH/22 Purified bulk (400 ng)

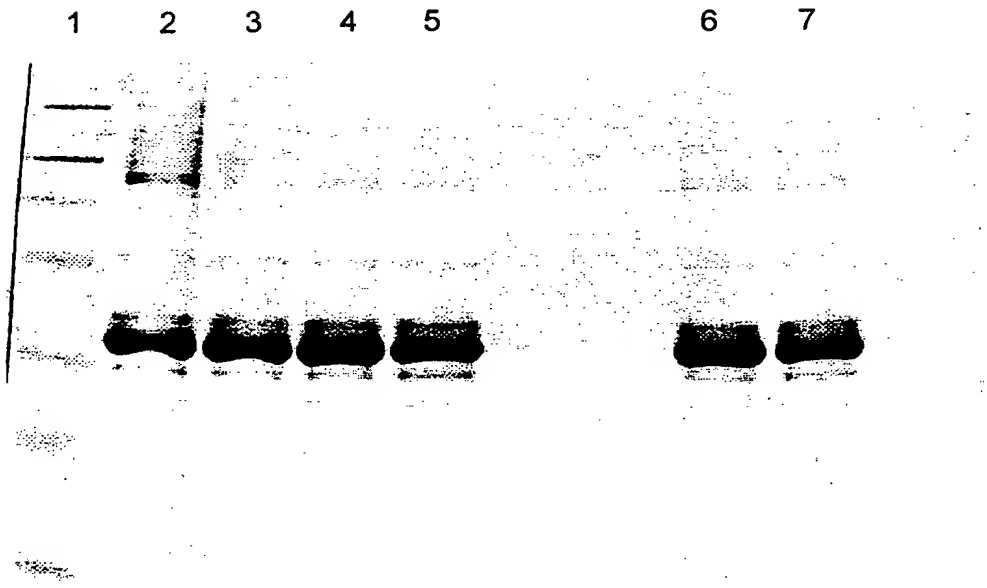
Daiichi Silver Staining



Blot Tat2

BlotαNef-Tat (LAS 97340)

Fig . 5 SDS-PAGE: Nef-Tat-his fusion protein



Coomassie blue G250

1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: TNH/23 SP eluate (4 µg)
3: TNH/23 Superdex200 eluate (4 µg)
4: TNH/23 Purified bulk (4 µg)
5: TNH/22 Purified bulk (4 µg)

6: TNH/23 Purified bulk (4 µg) / non reducing conditions
7: TNH/22 Purified bulk (4 µg) / non reducing conditions

Fig. 6A Tat-specific antibody titers and isotypes

group	immunization	midpoint titers				ratio IgG1/IgG2a
		Ig	IgG1	IgG2a	IgG2b	
1	oxydized Tat	353557	135538	98771	98763	1,372
2	reduced Tat	252275	72087	76273	72014	0,945
3	oxydized Nef-Tat	246466	179616	60835	53563	2,953
4	reduced Nef-Tat	91726	73767	30948	20679	2,384
5	adjuvant only	<4000	<4000	<4000	<4000	

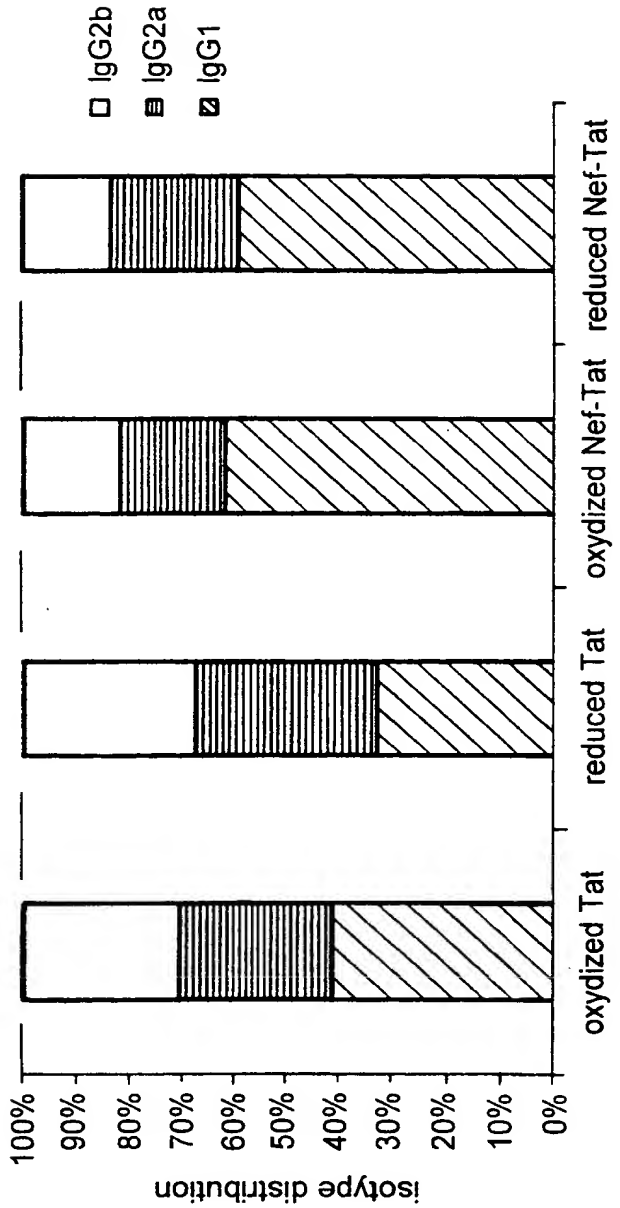


Fig. 6B Tat-specific antibody titers and isotypes

group	immunization	midpoint titers				ratio IgG1/IgG2a
		Ig	IgG1	IgG2a	IgG2b	
1	reduced Tat	212799	123242	62697	55763	1,966
2	reduced Nef-Tat	75676	84046	18449	11692	4,556
3	adjuvant only	<4000	<4000	<4000	<4000	

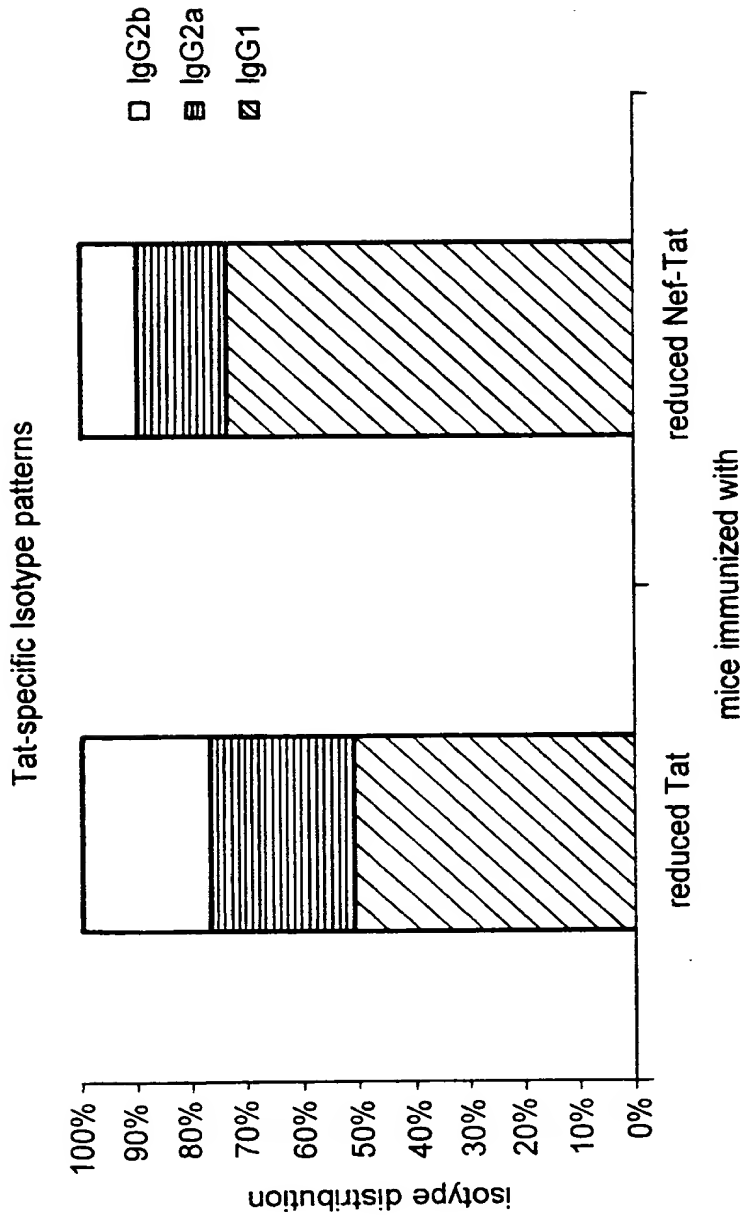


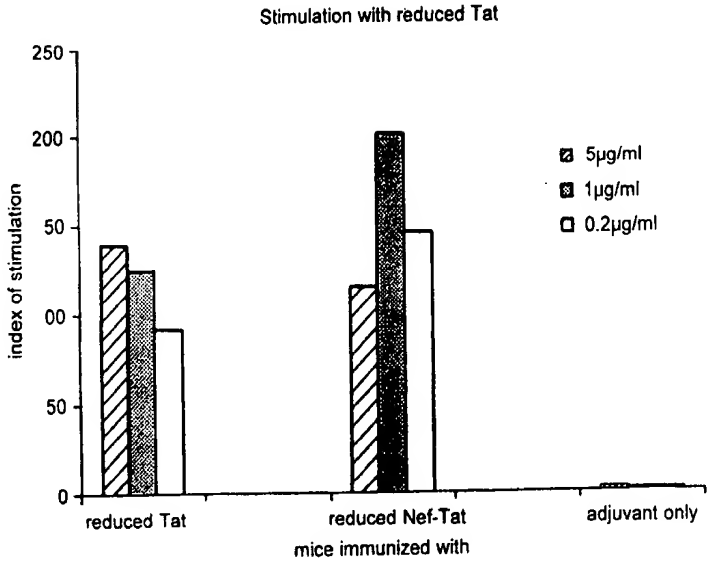
Fig. 7 Antigen-specific lymphoproliferative response of pooled lymph node cells

[3H] Thymidine incorporation in cpm

	Group 1 reduced Tat	Group 2 reduced Nef-Tat	Group 3 adjuvant only
reduced Tat			
5µg/ml	41967	18511	789
1µg/ml	37609	32346	415
0.2µg/ml	27640	23408	397
reduced Nef-Tat			
5µg/ml	43882	31694	483
1µg/ml	33865	28094	245
0.2µg/ml	25079	22891	383
medium	300	161	571

Data expressed as stimulation index

	Group 1 reduced Tat	Group 2 reduced Nef-Tat	Group 3 adjuvant only
reduced Tat			
5µg/ml	140	115	1
1µg/ml	125	201	1
0.2µg/ml	92	145	1
reduced Nef-Tat			
5µg/ml	146	197	1
1µg/ml	113	174	0
0.2µg/ml	84	142	1
medium	1	1	1



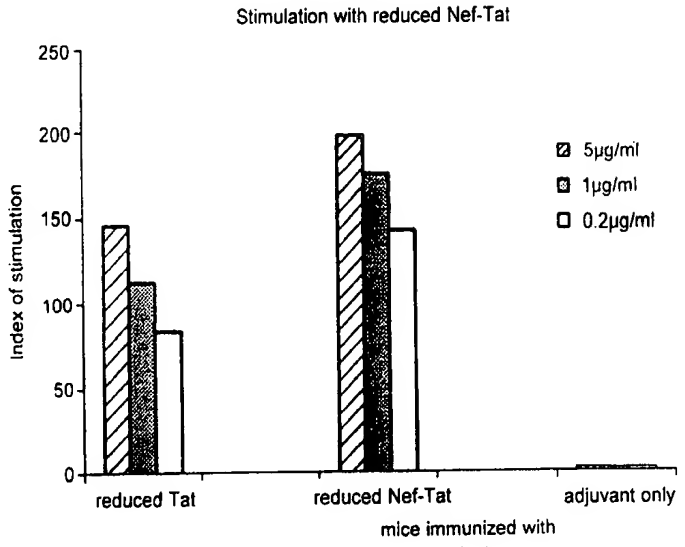
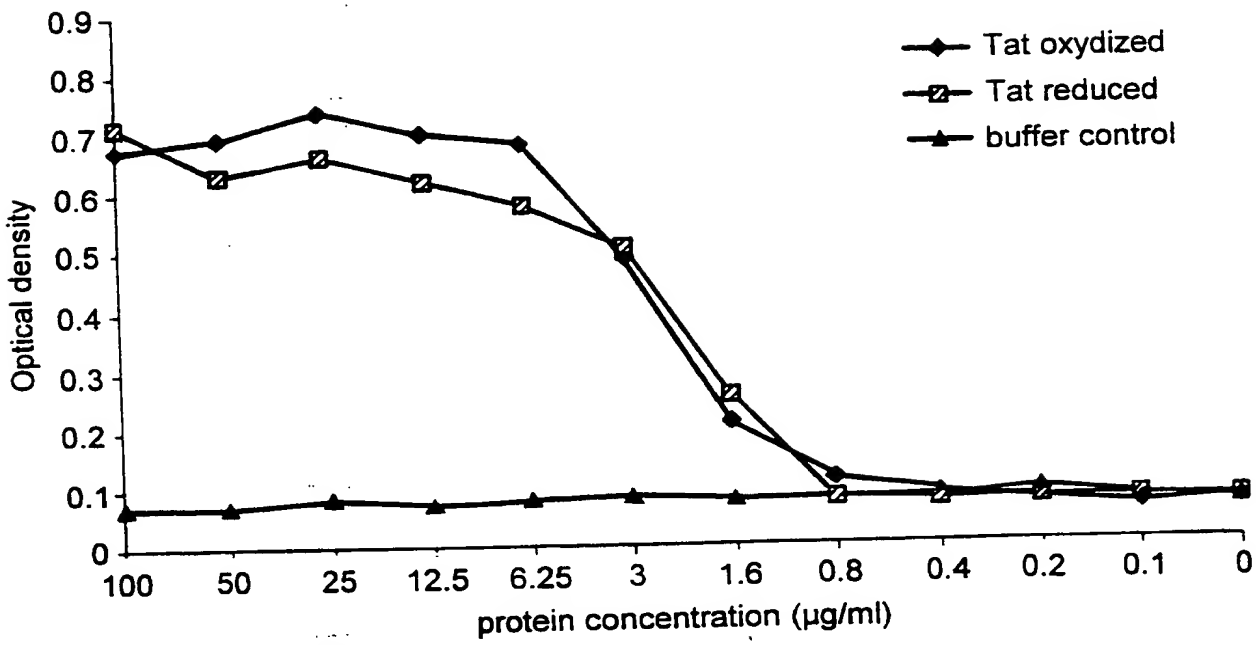


Fig. 8 Cell binding assay



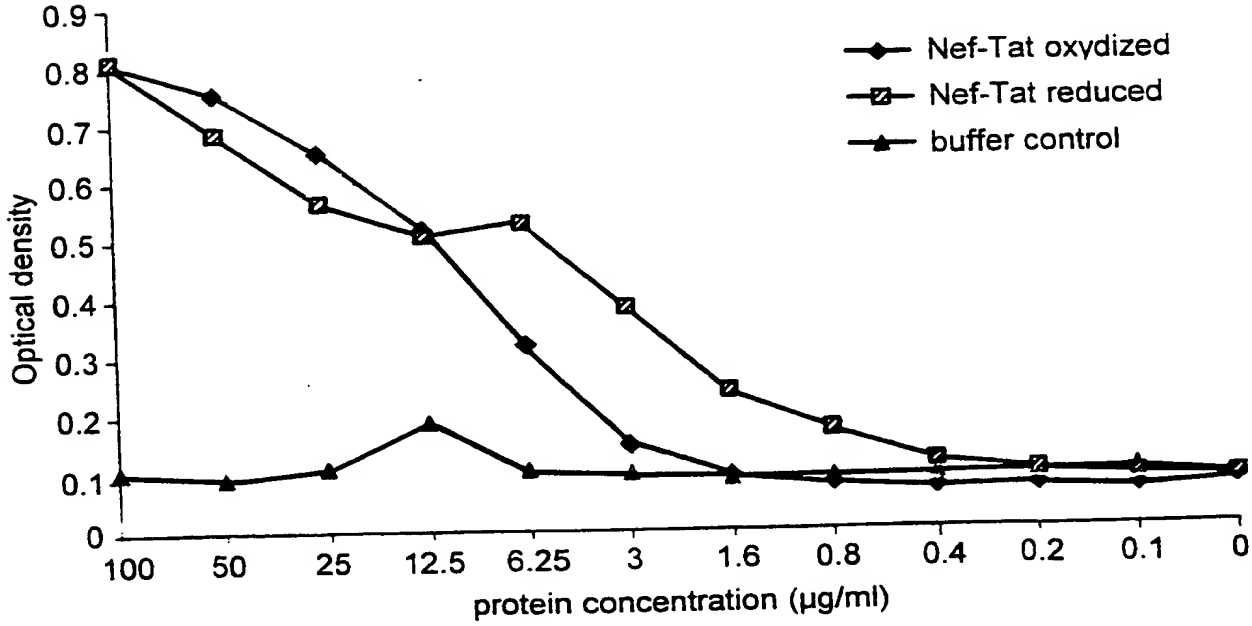
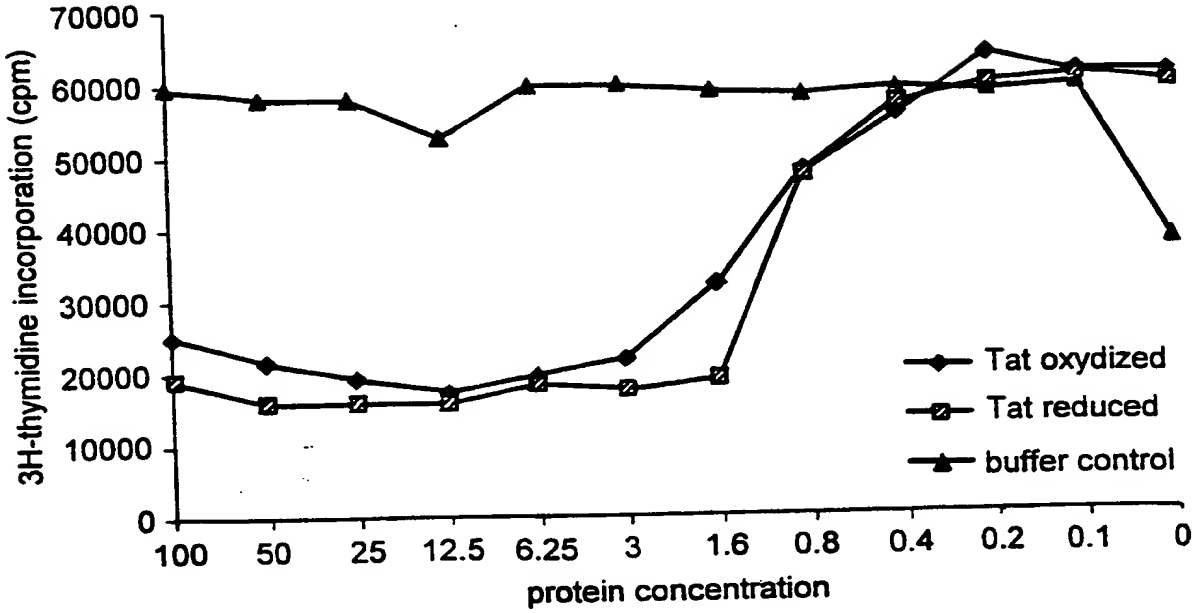
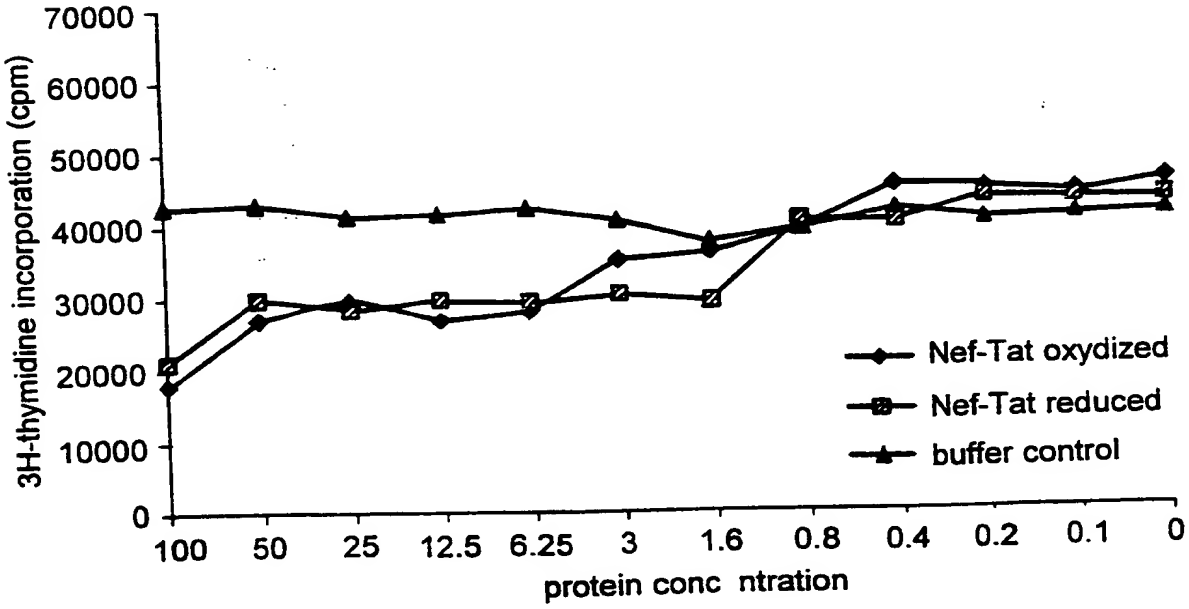


Fig. 9 Inhibition of cell growth





SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: SmithKline Beecham Biologicals S.A.

(ii) TITLE OF THE INVENTION: Vaccine

(iii) NUMBER OF SEQUENCES: 27

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: SmithKline Beecham
(B) STREET: Two New Horizons Court
(C) CITY: Brentford
(D) STATE:
(E) COUNTRY: Middx, UK
(F) ZIP: TW8 9EP

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 26-SEP-1997
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Bor, Fiona R
(B) REGISTRATION NUMBER:
(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 0181 975 2817
(B) TELEFAX: 0181 975 6141
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATCGTCCATG .GGT.GGC.A AG.TGG.T 28

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGGCTACTAG TGCAGTTCTT GAA 23

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATCGTACTAG T.GAG.CCA. GTA.GAT.C 29

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGCTACTAG TTTCCTTCGG GCCT 24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATCGTCCATG GAGCCAGTAG ATC 23

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 441 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
ATGGATCCAA AAACTTTAGC CCTTTCTTTA TTAGCAGCTG GCGTACTAGC AGGTTGTAGC     60
AGCCATTCAT CAAATATGGC GAATACCCAA ATGAAATCAG ACAAAATCAT TATTGCTCAC    120
CGTGGTGCTA GCGGTTATTT ACCAGAGCAT ACGTTAGAAT CTAAAGCACT TGCTTTTGCA    180
CAACAGGCTG ATTATTTAGA GCAAGATTTA GCAATGACTA AGGATGGTCG TTTAGTGGTT    240
ATTCACGATC ACTTTTTAGA TGGCTTGACT GATGTTGCGA AAAAATTCCC ACATCGTCAT    300
CGTAAAGATG GCCGTTACTA TGTCATCGAC TTTACCTTAA AAGAAATTCA AAGTTTAGAA    360
ATGACAGAAA ACTTTGAAAC CATGGCCACG TGTGATCAGA GCTCAACTAG TGGCCACCAT    420
CACCATCACC ATTAATCTAG A                                              441
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 144 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Met Asp Pro Lys Thr Leu Ala Leu Ser Leu Leu Ala Ala Gly Val Leu
 1               5                  10                  15
Ala Gly Cys Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
            20                  25                  30
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
        35                  40                  45
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
    50                  55                  60
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
65                  70                  75                  80
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
                85                  90                  95
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
            100                 105                 110
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
        115                 120                 125
Ala Thr Cys Asp Gln Ser Ser Thr Ser Gly His His His His His His
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 648 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGGGTGGCA AGTGGTCAAA AAGTAGTGTG GTTGGATGGC CTACTGTAAG GGAAAGAATG 60
AGACGAGCTG AGCCAGCAGC AGATGGGGTG GGAGCAGCAT CTCGAGACCT GGAAAAACAT 120
GGAGCAATCA CAAGTAGCAA TACAGCAGCT ACCAATGCTG CTTGTGCCTG GCTAGAAGCA 180
CAAGAGGAGG AGGAGGTGGG TTTTCCAGTC ACACCTCAGG TACCTTTAAG ACCAATGACT 240
TACAAGGCAG CTGTAGATCT TAGCCACTTT TTAAAAGAAA AGGGGGGACT GGAAGGGCTA 300
ATTCACTCCC AACGAAGACA AGATATCCTT GATCTGTGGA TCTACCACAC ACAAGGCTAC 360
TTCCCTGATT GGCAGAACTA CACACCAGGG CCAGGGGTCA GATATCCACT GACCTTTGGA 420
TGGTGCTACA AGCTAGTACC AGTTGAGCCA GATAAGGTAG AAGAGGCCAA TAAAGGAGAG 480
AACACCAGCT TGTTACACCC TGTGAGCCTG CATGGAATGG ATGACCCTGA GAGAGAAGTG 540
TTAGAGTGGA GGTTTGACAG CCGCCTAGCA TTTCATCACG TGGCCCGAGA GCTGCATCCG 600
GAGTACTTCA AGAACTGCAC TAGTGGCCAC CATCACCATC ACCATTAA 648

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 216 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val
1 5 10 15
Arg Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala
20 25 30
Ala Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr
35 40 45
Ala Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu
50 55 60
Glu Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr
65 70 75 80
Tyr Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly
85 90 95
Leu Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu
100 105 110
Trp Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr
115 120 125
Pro Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys
130 135 140
Leu Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu
145 150 155 160
Asn Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro
165 170 175
Glu Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His
180 185 190
His Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser
195 200 205
Gly His His His His His His
210 215

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 288 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
ATGGAGCCAG TAGATCCTAG ACTAGAGCCC TGGAAGCATC CAGGAAGTCA GCCTAAAACT     60
GCTTGTACCA ATTGCTATTG TAAAAAGTGT TGCTTTCATT GCCAAGTTTG TTTCATAACA    120
AAAGCCTTAG GCATCTCCTA TGGCAGGAAG AAGCGGAGAC AGCGACGAAG ACCTCCTCAA    180
GGCAGTCAGA CTCATCAAGT TTCTCTATCA AAGCAACCCA CCTCCCAATC CCGAGGGGAC    240
CCGACAGGCC CGAAGGAAAC TAGTGGCCAC CATCACCATC ACCATTAA                 288
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Met Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser
 1               5                  10                  15
Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe
            20                  25                  30
His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly
        35                  40                  45
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr
    50                  55                  60
His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp
65                  70                  75                  80
Pro Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His
                85                  90                  95
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 909 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
ATGGGTGGCA AGTGGTCAAA AAGTAGTGTG GTTGGATGGC CTACTGTAAG GGAAAGAATG     60
AGACGAGCTG AGCCAGCAGC AGATGGGGTG GGAGCAGCAT CTCGAGACCT GGAAAAACAT    120
GGAGCAATCA CAAGTAGCAA TACAGCAGCT ACCAATGCTG CTTGTGCCTG GCTAGAAGCA    180
CAAGAGGAGG AGGAGGTGGG TTTTCCAGTC ACACCTCAGG TACCTTTAAG ACCAATGACT    240
TACAAGGCAG CTGTAGATCT TAGCCACTTT TTAAAAGAAA AGGGGGGACT GGAAGGGCTA    300
ATTCACTCCC AACGAAGACA AGATATCCTT GATCTGTGGA TCTACCACAC ACAAGGCTAC    360
```

```
TTCCCTGATT GGCAGAACTA CACACCAGGG CCAGGGGTCA GATATCCACT GACCTTTGGA    420
TGGTGCTACA AGCTAGTACC AGTTGAGCCA GATAAGGTAG AAGAGGCCAA TAAAGGAGAG    480
AACACCAGCT TGTTACACCC TGTGAGCCTG CATGGAATGG ATGACCCTGA GAGAGAAGTG    540
TTAGAGTGGA GGTTTGACAG CCGCCTAGCA TTTCATCACG TGGCCCGAGA GCTGCATCCG    600
GAGTACTTCA AGAACTGCAC TAGTGAGCCA GTAGATCCTA GACTAGAGCC CTGGAAGCAT    660
CCAGGAAGTC AGCCTAAAAC TGCTTGTACC AATTGCTATT GTAAAAAGTG TTGCTTTCAT    720
TGCCAAGTTT GTTTCATAAC AAAAGCCTTA GGCATCTCCT ATGGCAGGAA GAAGCGGAGA    780
CAGCGACGAA GACCTCCTCA AGGCAGTCAG ACTCATCAAG TTTCTCTATC AAAGCAACCC    840
ACCTCCCAAT CCCGAGGGGA CCCGACAGGC CCGAAGGAAA CTAGTGGCCA CCATCACCAT    900
CACCATTAA                                                            909
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 303 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Met Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val
 1               5                  10                  15
Arg Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala
            20                  25                  30
Ala Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr
        35                  40                  45
Ala Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu
    50                  55                  60
Glu Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr
65                  70                  75                  80
Tyr Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly
                85                  90                  95
Leu Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu
            100                 105                 110
Trp Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr
        115                 120                 125
Pro Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys
    130                 135                 140
Leu Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu
145                 150                 155                 160
Asn Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro
                165                 170                 175
Glu Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His
            180                 185                 190
His Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser
        195                 200                 205
Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln
    210                 215                 220
Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His
225                 230                 235                 240
Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg
                245                 250                 255
Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His
            260                 265                 270
Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro
```

```
        275                 280                 285
Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His
    290                 295                 300
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1029 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
ATGGATCCAA AAACTTTAGC CCTTTCTTTA TTAGCAGCTG GCGTACTAGC AGGTTGTAGC     60
AGCCATTCAT CAAATATGGC GAATACCCAA ATGAAATCAG ACAAAATCAT TATTGCTCAC    120
CGTGGTGCTA GCGGTTATTT ACCAGAGCAT ACGTTAGAAT CTAAAGCACT TGCTTTTGCA    180
CAACAGGCTG ATTATTTAGA GCAAGATTTA GCAATGACTA AGGATGGTCG TTTAGTGGTT    240
ATTCACGATC ACTTTTTAGA TGGCTTGACT GATGTTGCGA AAAAATTCCC ACATCGTCAT    300
CGTAAAGATG GCCGTTACTA TGTCATCGAC TTTACCTTAA AAGAAATTCA AAGTTTAGAA    360
ATGACAGAAA ACTTTGAAAC CATGGGTGGC AAGTGGTCAA AAAGTAGTGT GGTTGGATGG    420
CCTACTGTAA GGGAAAGAAT GAGACGAGCT GAGCCAGCAG CAGATGGGGT GGGAGCAGCA    480
TCTCGAGACC TGGAAAAACA TGGAGCAATC ACAAGTAGCA ATACAGCAGC TACCAATGCT    540
GCTTGTGCCT GGCTAGAAGC ACAAGAGGAG GAGGAGGTGG GTTTTCCAGT CACACCTCAG    600
GTACCTTTAA GACCAATGAC TTACAAGGCA GCTGTAGATC TTAGCCACTT TTTAAAAGAA    660
AAGGGGGGAC TGGAAGGGCT AATTCACTCC CAACGAAGAC AAGATATCCT TGATCTGTGG    720
ATCTACCACA CACAAGGCTA CTTCCCTGAT TGGCAGAACT ACACACCAGG GCCAGGGGTC    780
AGATATCCAC TGACCTTTGG ATGGTGCTAC AAGCTAGTAC CAGTTGAGCC AGATAAGGTA    840
GAAGAGGCCA ATAAAGGAGA GAACACCAGC TTGTTACACC CTGTGAGCCT GCATGGAATG    900
GATGACCCTG AGAGAGAAGT GTTAGAGTGG AGGTTTGACA GCCGCCTAGC ATTTCATCAC    960
GTGGCCCGAG AGCTGCATCC GGAGTACTTC AAGAACTGCA CTAGTGGCCA CCATCACCAT   1020
CACCATTAA                                                           1029
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 325 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Cys Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys Ser Asp
 1               5                  10                  15
Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro Glu His
            20                  25                  30
Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp Tyr Leu
        35                  40                  45
Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val Ile His
    50                  55                  60
Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe Pro His
65                  70                  75                  80
Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr Leu Lys
                85                  90                  95
```

```
Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met Gly Gly
          100                 105                 110
Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg Glu Arg
      115                 120                 125
Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala Ser Arg
  130                 135                 140
Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala Ala Thr
145                 150                 155                 160
Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Glu Val Gly
                165                 170                 175
Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr Lys Ala
            180                 185                 190
Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu Glu Gly
        195                 200                 205
Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp Ile Tyr
    210                 215                 220
His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro Gly Pro
225                 230                 235                 240
Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu Val Pro
                245                 250                 255
Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn Thr Ser
            260                 265                 270
Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu Arg Glu
        275                 280                 285
Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His Val Ala
    290                 295                 300
Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Gly His His
305                 310                 315                 320
His His His His
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1290 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
ATGGATCCAA AAACTTTAGC CCTTTCTTTA TTAGCAGCTG GCGTACTAGC AGGTTGTAGC     60
AGCCATTCAT CAAATATGGC GAATACCCAA ATGAAATCAG ACAAAATCAT TATTGCTCAC    120
CGTGGTGCTA GCGGTTATTT ACCAGAGCAT ACGTTAGAAT CTAAAGCACT TGCGTTTGCA    180
CAACAGGCTG ATTATTTAGA GCAAGATTTA GCAATGACTA AGGATGGTCG TTTAGTGGTT    240
ATTCACGATC ACTTTTTAGA TGGCTTGACT GATGTTGCGA AAAAATTCCC ACATCGTCAT    300
CGTAAAGATG GCCGTTACTA TGTCATCGAC TTTACCTTAA AAGAAATTCA AAGTTTAGAA    360
ATGACAGAAA ACTTTGAAAC CATGGGTGGC AAGTGGTCAA AAAGTAGTGT GGTTGGATGG    420
CCTACTGTAA GGGAAAGAAT GAGACGAGCT GAGCCAGCAG CAGATGGGGT GGGAGCAGCA    480
TCTCGAGACC TGGAAAAACA TGGAGCAATC ACAAGTAGCA ATACAGCAGC TACCAATGCT    540
GCTTGTGCCT GGCTAGAAGC ACAAGAGGAG GAGGAGGTGG GTTTTCCAGT CACACCTCAG    600
GTACCTTTAA GACCAATGAC TTACAAGGCA GCTGTAGATC TTAGCCACTT TTTAAAAGAA    660
AAGGGGGGAC TGGAAGGGCT AATTCACTCC CAACGAAGAC AAGATATCCT TGATCTGTGG    720
ATCTACCACA CACAAGGCTA CTTCCCTGAT TGGCAGAACT ACACACCAGG GCCAGGGGTC    780
AGATATCCAC TGACCTTTGG ATGGTGCTAC AAGCTAGTAC CAGTTGAGCC AGATAAGGTA    840
GAAGAGGCCA ATAAAGGAGA GAACACCAGC TTGTTACACC CTGTGAGCCT GCATGGAATG    900
```

```
GATGACCCTG AGAGAGAAGT GTTAGAGTGG AGGTTTGACA GCCGCCTAGC ATTTCATCAC    960
GTGGCCCGAG AGCTGCATCC GGAGTACTTC AAGAACTGCA CTAGTGAGCC AGTAGATCCT   1020
AGACTAGAGC CCTGGAAGCA TCCAGGAAGT CAGCCTAAAA CTGCTTGTAC CAATTGCTAT   1080
TGTAAAAAGT GTTGCTTTCA TTGCCAAGTT TGTTTCATAA CAAAAGCCTT AGGCATCTCC   1140
TATGGCAGGA AGAAGCGGAG ACAGCGACGA AGACCTCCTC AAGGCAGTCA GACTCATCAA   1200
GTTTCTCTAT CAAAGCAACC CACCTCCCAA TCCCGAGGGG ACCCGACAGG CCCGAAGGAA   1260
ACTAGTGGCC ACCATCACCA TCACCATTAA                                    1290
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 412 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Cys Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys Ser Asp
 1               5                  10                  15
Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro Glu His
            20                  25                  30
Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp Tyr Leu
        35                  40                  45
Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val Ile His
    50                  55                  60
Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe Pro His
65                  70                  75                  80
Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr Leu Lys
                85                  90                  95
Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met Gly Gly
            100                 105                 110
Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg Glu Arg
        115                 120                 125
Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala Ser Arg
    130                 135                 140
Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala Ala Thr
145                 150                 155                 160
Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Glu Val Gly
                165                 170                 175
Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr Lys Ala
            180                 185                 190
Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu Glu Gly
        195                 200                 205
Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp Ile Tyr
    210                 215                 220
His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro Gly Pro
225                 230                 235                 240
Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu Val Pro
                245                 250                 255
Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn Thr Ser
            260                 265                 270
Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu Arg Glu
        275                 280                 285
Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His Val Ala
    290                 295                 300
```

```
Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Glu Pro Val
305                 310                 315                 320
Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro Lys Thr
                325                 330                 335
Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His Cys Gln Val
            340                 345                 350
Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys Lys Arg
        355                 360                 365
Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His Gln Val Ser
    370                 375                 380
Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro Thr Gly Pro
385                 390                 395                 400
Lys Glu Thr Ser Gly His His His His His His
                405                 410
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 981 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC AGACAAAATC     60
ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC ATACGTTAGA ATCTAAAGCA    120
CTTGCGTTTG CACAACAGGC TGATTATTTA GAGCAAGATT TAGCAATGAC TAAGGATGGT    180
CGTTTAGTGG TTATTCACGA TCACTTTTTA GATGGCTTGA CTGATGTTGC GAAAAAATTC    240
CCACATCGTC ATCGTAAAGA TGGCCGTTAC TATGTCATCG ACTTTACCTT AAAAGAAATT    300
CAAAGTTTAG AAATGACAGA AAACTTTGAA ACCATGGGTG GCAAGTGGTC AAAAAGTAGT    360
GTGGTTGGAT GGCCTACTGT AAGGGAAAGA ATGAGACGAG CTGAGCCAGC AGCAGATGGG    420
GTGGGAGCAG CATCTCGAGA CCTGGAAAAA CATGGAGCAA TCACAAGTAG CAATACAGCA    480
GCTACCAATG CTGCTTGTGC CTGGCTAGAA GCACAAGAGG AGGAGGAGGT GGGTTTTCCA    540
GTCACACCTC AGGTACCTTT AAGACCAATG ACTTACAAGG CAGCTGTAGA TCTTAGCCAC    600
TTTTTAAAAG AAAAGGGGGG ACTGGAAGGG CTAATTCACT CCCAACGAAG ACAAGATATC    660
CTTGATCTGT GGATCTACCA CACACAAGGC TACTTCCCTG ATTGGCAGAA CTACACACCA    720
GGGCCAGGGG TCAGATATCC ACTGACCTTT GGATGGTGCT ACAAGCTAGT ACCAGTTGAG    780
CCAGATAAGG TAGAAGAGGC CAATAAAGGA GAGAACACCA GCTTGTTACA CCCTGTGAGC    840
CTGCATGGAA TGGATGACCC TGAGAGAGAA GTGTTAGAGT GGAGGTTTGA CAGCCGCCTA    900
GCATTTCATC ACGTGGCCCG AGAGCTGCAT CCGGAGTACT TCAAGAACTG CACTAGTGGC    960
CACCATCACC ATCACCATTA A                                              981
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 327 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
 1               5                  10                  15
```

```
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
            20                  25                  30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
        35                  40                  45
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
    50                  55                  60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65                  70                  75                  80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
                85                  90                  95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
            100                 105                 110
Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg
        115                 120                 125
Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala
    130                 135                 140
Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala
145                 150                 155                 160
Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Glu
                165                 170                 175
Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr
            180                 185                 190
Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu
        195                 200                 205
Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp
    210                 215                 220
Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro
225                 230                 235                 240
Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu
                245                 250                 255
Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn
            260                 265                 270
Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu
        275                 280                 285
Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His
    290                 295                 300
Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Gly
305                 310                 315                 320
His His His His His His
                325
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1242 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC AGACAAAATC     60
ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC ATACGTTAGA ATCTAAAGCA    120
CTTGCGTTTG CACAACAGGC TGATTATTTA GAGCAAGATT TAGCAATGAC TAAGGATGGT    180
CGTTTAGTGG TTATTCACGA TCACTTTTTA GATGGCTTGA CTGATGTTGC GAAAAAATTC    240
CCACATCGTC ATCGTAAAGA TGGCCGTTAC TATGTCATCG ACTTTACCTT AAAAGAAATT    300
```

```
CAAAGTTTAG AAATGACAGA AAACTTTGAA ACCATGGGTG GCAAGTGGTC AAAAAGTAGT    360
GTGGTTGGAT GGCCTACTGT AAGGGAAAGA ATGAGACGAG CTGAGCCAGC AGCAGATGGG    420
GTGGGAGCAG CATCTCGAGA CCTGGAAAAA CATGGAGCAA TCACAAGTAG CAATACAGCA    480
GCTACCAATG CTGCTTGTGC CTGGCTAGAA GCACAAGAGG AGGAGGAGGT GGGTTTTCCA    540
GTCACACCTC AGGTACCTTT AAGACCAATG ACTTACAAGG CAGCTGTAGA TCTTAGCCAC    600
TTTTTAAAAG AAAAGGGGGG ACTGGAAGGG CTAATTCACT CCCAACGAAG ACAAGATATC    660
CTTGATCTGT GGATCTACCA CACACAAGGC TACTTCCCTG ATTGGCAGAA CTACACACCA    720
GGGCCAGGGG TCAGATATCC ACTGACCTTT GGATGGTGCT ACAAGCTAGT ACCAGTTGAG    780
CCAGATAAGG TAGAAGAGGC CAATAAAGGA GAGAACACCA GCTTGTTACA CCCTGTGAGC    840
CTGCATGGAA TGGATGACCC TGAGAGAGAA GTGTTAGAGT GGAGGTTTGA CAGCCGCCTA    900
GCATTTCATC ACGTGGCCCG AGAGCTGCAT CCGGAGTACT TCAAGAACTG CACTAGTGAG    960
CCAGTAGATC CTAGACTAGA GCCCTGGAAG CATCCAGGAA GTCAGCCTAA AACTGCTTGT   1020
ACCAATTGCT ATTGTAAAAA GTGTTGCTTT CATTGCCAAG TTTGTTTCAT AACAAAAGCC   1080
TTAGGCATCT CCTATGGCAG GAAGAAGCGG AGACAGCGAC GAAGACCTCC TCAAGGCAGT   1140
CAGACTCATC AAGTTTCTCT ATCAAAGCAA CCCACCTCCC AATCCCGAGG GGACCCGACA   1200
GGCCCGAAGG AAACTAGTGG CCACCATCAC CATCACCATT AA                       1242
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 414 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
 1               5                  10                  15
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
            20                  25                  30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
        35                  40                  45
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
    50                  55                  60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65                  70                  75                  80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
                85                  90                  95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
            100                 105                 110
Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg
        115                 120                 125
Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala
    130                 135                 140
Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala
145                 150                 155                 160
Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Glu
                165                 170                 175
Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr
            180                 185                 190
Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu
        195                 200                 205
Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp
    210                 215                 220
Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro
```

```
225                 230                 235                 240
Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu
                245                 250                 255
Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn
            260                 265                 270
Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu
        275                 280                 285
Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His
    290                 295                 300
Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Glu
305                 310                 315                 320
Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro
                325                 330                 335
Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His Cys
            340                 345                 350
Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys
        355                 360                 365
Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His Gln
    370                 375                 380
Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro Thr
385                 390                 395                 400
Gly Pro Lys Glu Thr Ser Gly His His His His His His
                405                 410
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 288 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
ATGGAGCCAG TAGATCCTAG ACTAGAGCCC TGGAAGCATC CAGGAAGTCA GCCTAAAACT     60
GCTTGTACCA ATTGCTATTG TAAAAAGTGT TGCTTTCATT GCCAAGTTTG TTTCATAACA    120
GCTGCCTTAG GCATCTCCTA TGGCAGGAAG AAGCGGAGAC AGCGACGAAG ACCTCCTCAA    180
GGCAGTCAGA CTCATCAAGT TTCTCTATCA AAGCAACCCA CCTCCCAATC CAAAGGGGAG    240
CCGACAGGCC CGAAGGAAAC TAGTGGCCAC CATCACCATC ACCATTAA                 288
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
Met Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser
 1               5                  10                  15
Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe
            20                  25                  30
His Cys Gln Val Cys Phe Ile Thr Ala Ala Leu Gly Ile Ser Tyr Gly
```

```
        35                  40                  45
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr
    50                  55                  60
His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Lys Gly Glu
65                  70                  75                  80
Pro Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His
                85                  90                  95
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 909 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
ATGGGTGGCA AGTGGTCAAA AAGTAGTGTG GTTGGATGGC CTACTGTAAG GGAAAGAATG     60
AGACGAGCTG AGCCAGCAGC AGATGGGGTG GGAGCAGCAT CTCGAGACCT GGAAAAACAT    120
GGAGCAATCA CAAGTAGCAA TACAGCAGCT ACCAATGCTG CTTGTGCCTG GCTAGAAGCA    180
CAAGAGGAGG AGGAGGTGGG TTTTCCAGTC ACACCTCAGG TACCTTTAAG ACCAATGACT    240
TACAAGGCAG CTGTAGATCT TAGCCACTTT TTAAAAGAAA AGGGGGGACT GGAAGGGCTA    300
ATTCACTCCC AACGAAGACA AGATATCCTT GATCTGTGGA TCTACCACAC ACAAGGCTAC    360
TTCCCTGATT GGCAGAACTA CACACCAGGG CCAGGGGTCA GATATCCACT GACCTTTGGA    420
TGGTGCTACA AGCTAGTACC AGTTGAGCCA GATAAGGTAG AAGAGGCCAA TAAAGGAGAG    480
AACACCAGCT TGTTACACCC TGTGAGCCTG CATGGAATGG ATGACCCTGA GAGAGAAGTG    540
TTAGAGTGGA GGTTTGACAG CCGCCTAGCA TTTCATCACG TGGCCCGAGA GCTGCATCCG    600
GAGTACTTCA AGAACTGCAC TAGTGAGCCA GTAGATCCTA GACTAGAGCC CTGGAAGCAT    660
CCAGGAAGTC AGCCTAAAAC TGCTTGTACC AATTGCTATT GTAAAAAGTG TTGCTTTCAT    720
TGCCAAGTTT GTTTCATAAC AGCTGCCTTA GGCATCTCCT ATGGCAGGAA GAAGCGGAGA    780
CAGCGACGAA GACCTCCTCA AGGCAGTCAG ACTCATCAAG TTTCTCTATC AAAGCAACCC    840
ACCTCCCAAT CCAAAGGGGA GCCGACAGGC CCGAAGGAAA CTAGTGGCCA CCATCACCAT    900
CACCATTAA                                                           909
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 303 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
Met Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val
 1               5                  10                  15
Arg Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala
            20                  25                  30
Ala Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr
        35                  40                  45
Ala Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu
    50                  55                  60
Glu Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr
65                  70                  75                  80
```

```
Tyr Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly
                85                  90                  95
Leu Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu
            100                 105                 110
Trp Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr
        115                 120                 125
Pro Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys
    130                 135                 140
Leu Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu
145                 150                 155                 160
Asn Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro
                165                 170                 175
Glu Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His
            180                 185                 190
His Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser
        195                 200                 205
Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln
    210                 215                 220
Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His
225                 230                 235                 240
Cys Gln Val Cys Phe Ile Thr Ala Ala Leu Gly Ile Ser Tyr Gly Arg
                245                 250                 255
Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His
            260                 265                 270
Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Lys Gly Glu Pro
        275                 280                 285
Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His
    290                 295                 300
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTCGAAACCA TGGCCGCGGA CTAGTGGCCA CCATCACCAT CACCATTAAC GGAATTC 57

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
Thr Ser Gly His His His His His His
 1               5
```

INTERNATIONAL SEARCH REPORT

Inter[illegible]nal Application No
PCT/EP 98/06040

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/49 C12N15/62 C07K14/16 A61K39/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 04686 A (BARSOUM JAMES G ;BIOGEN INC (US); FAWELL STEPHEN E (US); PEPINSKY) 3 March 1994 see page 54 - page 73 ---	1,4, 13-15
X	BODÉUS M ET AL.: "In vitro binding and phosphorylation of human immunodefciency virus type 1 Nef protein by serine/threonine protein kinase" JOURNAL OF GENERAL VIROLOGY, vol. 76, no. 6, June 1995, pages 1337-1344, XP002092508 READING GB see page 1338, left-hand column, paragraph 3 --- -/--	1,5, 13-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 February 1999	18/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

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INTERNATIONAL SEARCH REPORT

Inter[illegible]nal Application No
PCT/EP 98/06040

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SALFELD J ET AL: "A tripartite HIV-1 tat-env-rev fusion protein" EMBO JOURNAL, vol. 9, no. 3, 1 March 1990, pages 965-970, XP000113784 see the whole document ---	1,4
X	AHMED A AZAD ET AL: "Large-scale production and characterization of recombinant human immunodeficiency virus type 1 Nef" JOURNAL OF GENERAL VIROLOGY, vol. 75, no. 3, 1 March 1994, pages 651-655, XP000565729 see the whole document ---	1,5, 13-15
A	JANSON H ET AL.: "Protein D, the immunoglobulin D-binding protein of Haemophilus influenzae, is a lipoprotein" INFECTION AND IMMUNITY, vol. 60, no. 4, April 1992, pages 1336-1342, XP002092509 WASHINGTON US cited in the application see the whole document -----	6-8

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 98/06040

Patent document cited in search report	Publication date	Patent family member(s)	Publication dat
WO 9404686 A	03-03-1994	AT 173016 T	15-11-1998
		AU 667244 B	14-03-1996
		AU 5083293 A	15-03-1994
		CA 2135642 A	03-03-1994
		DE 69321962 D	10-12-1998
		DE 656950 T	14-03-1996
		EP 0656950 A	14-06-1995
		ES 2123062 T	01-01-1999
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		JP 10033186 A	10-02-1998
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		US 5670617 A	23-09-1997
		US 5652122 A	29-07-1997
		US 5747641 A	05-05-1998

CLAIMS

1. A protein comprising

(a) an HIV Tat protein or derivative thereof linked to either (i) a fusion partner or (ii) an HIV Nef protein or derivative thereof; or

(b) an HIV Nef protein or derivative thereof linked to either (i) a fusion partner or (ii) an HIV Tat protein or derivative thereof; or

(c) an HIV Nef protein or derivative thereof linked to an HIV Tat protein or derivative thereof and a fusion partner.

2. A protein as claimed in claim 1 which is a Tat-Nef fusion protein or derivative thereof.

3. A protein as claimed in claim 1 which is a Nef-Tat fusion protein or derivative thereof.

4. A protein according to claim 1 wherein the derivative of the Tat protein is a mutated Tat protein.

5. A protein according to claim 1 wherein the derivative of the Nef protein is a mutated Nef protein.

6. A Protein as claimed in any one of claims 1 - 5 wherein the fusion partner is a lipoprotein or derivative thereof.

7. A protein as claimed in claim 6 wherein the lipoprotein is Haemophilus Influenza B protein D or derivative thereof.

8. A protein as claimed in Claim 7 wherein the fusion partner comprises between 100-130 amino acid from the N terminal of Haemophilus Influenza B protein D.

9. A protein as claimed in any one of Claims 1 to 8, wherein the Tat protein is the entire Tat protein.

10. A protein as claimed in any one of Claims 1 to 8, wherein the Nef protein is the entire Nef protein.

11. A protein as claimed in any one of Claims 1 to 10, wherein the Tat protein is fused to an HIV Nef protein and a fusion partner.

12. A protein as claimed in any one of Claims 1 to 11, wherein the protein has a Histidine tail.

13. A nucleic acid encoding a protein of Claims 1 to 12.

14. A host transformed with a nucleic acid of Claim 13.

15. A host as claimed in claim 14 wherein the host is either Pichia pastoris or E. coli.

16. A vaccine comprising a protein of any one of Claims 1 to 12 in admixture with a pharmaceutically acceptable excipient.

17. A vaccine of Claim 16 additionally comprising an adjuvant.

18. A vaccine of claim 17 wherein the adjuvant is a TH1 inducing adjuvant.

19. A vaccine as claimed in Claim 17 or 18 which adjuvant comprises monophosphoryl lipid A or derivative thereof such as 3 de-O-acylated monophosphoryl lipid A.

20. A vaccine as claimed in any one of Claims 16 to 19 additionally comprising a saponin adjuvant.

21. A method of producing a protein of Claim 1 to 12, comprising the steps of transforming a host with a nucleic acid encoding said protein, expressing said protein and recovering the protein.

22. A method as claimed in Claim 21 wherein the host is E. coli. or *Pichia pastoris*.

23. A method of producing a vaccine of Claim 16 to 20, comprising admixing the protein of Claim 1 to 12 with a pharmaceutically acceptable diluent.

24. A method of preparing (i) an HIV Nef protein or derivative thereof or (ii) an HIV Tat protein or derivative thereof in *Pichia pastoris* which method comprises the steps of transforming Pichia pastoris with DNA encoding said HIV Nef protein or derivative thereof or HIV Tat protein or derivative thereof, expressing said protein and recovering the protein.